The Handling of the Mechanistic Probe 5-Fluorouridine by the Pseudouridine Synthase TruA and Its Consistency with the Handling of the Same Probe by the Pseudouridine Synthases TruB and RluA[†]

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ABSTRACT: RNA containing 5-fluorouridine (F^5U) had previously been used to examine the mechanism of the pseudouridine synthase TruA, formerly known as pseudouridine synthase I [Gu et al. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 14270−14275]. From that work, it was reasonably concluded that the pseudouridine synthases proceed via a mechanism involving a Michael addition by an active site aspartic acid residue to the pyrimidine ring of uridine or F^5U. Those conclusions rested on the assumption that the hydrate of F^5U was obtained after digestion of the product RNA and that hydration resulted from hydrolysis of the ester intermediate between the aspartic acid residue and F^5U. As reported here, ^{18}O labeling definitively demonstrates that ester hydrolysis does not give rise to the observed hydrated product and that digestion generates not the expected mononucleoside product but rather a dinucleotide between a hydrated isomer of F^5U and the following nucleoside in RNA. The discovery that digestion products are dinucleotides accounts for the previously puzzling differences in the isolated products obtained following the action of the pseudouridine synthases TruB and RluA on F^5U in RNA.

The pseudouridine synthases (Ψ synthases)[1] catalyze the most common posttranscriptional modification in RNA, the isomerization of uridine to pseudouridine (Ψ). Based on sequence data, the Ψ synthases were grouped into six families that share no global statistically significant sequence similarity (*1*). Several conserved motifs were identified, however, and one included the only absolutely conserved amino acid residue, an Asp that is essential for activity (*2−6*). The structural elucidation of members of each family revealed that the Ψ synthases share the same overall fold and are thus related by divergent rather than convergent evolution (*1*).

Given their same overall fold and largely conserved constellation of active site residues (including the essential Asp), the families all likely follow the same catalytic mechanism. Two alternatives seem likely from chemical precedent and mechanistic work. In the first (Figure 1, panel A), the essential Asp nucleophilically attacks the pyrimidine ring in a Michael addition to make an ester intermediate (*7*). After breakage of the glycosidic bond, the pyrimidine ring rotates about the new ester bond to reposition C5 near C1′ to form the characteristic *C*-glycosidic linkage of Ψ. Deprotonation of C5 and protonation of N1 complete the reaction. In the second mechanism (Figure 1, panel B), the essential Asp nucleophilically attacks the ribose rather than the pyrimidine ring to form an acyl intermediate (*2*). The detached pyrimidine ring rotates to allow formation of a bond between C5 and C1′ to generate the *C*-glycoside, the deprotonation of which generates Ψ.

To examine the mechanism of the Ψ synthases, Santi and co-workers employed the mechanistic probe 5-fluorouridine (F^5U) in the *in vitro* transcript of *Escherichia coli* tRNA^{Phe} (abbreviated [F^5U]tRNA) and TruA (formerly called Ψ synthase I) (*2, 7*). These two species form a covalent adduct as judged by the irreversible inhibition of the enzyme and a shifted band containing both TruA and RNA in denaturing gels. The adduct was heat-disrupted, and the RNA was isolated. The mass spectrum of the oligonucleotides resulting from digestion of the RNA by RNase A (which cuts after U and C) clearly showed that F^5U had become hydrated. The RNA was also digested with nuclease P1 and alkaline phosphatase to generate the component nucleosides, which were examined by chromatography (for these experiments, tritiated F^5UTP was used to prepare the [F^5U]tRNA so that the small amount of material could be sensitively tracked). The component nucleosides were examined by cellulose thin-layer chromatography, and the nucleoside product of F^5U behaved very similarly to the two isomers of authentic hydrated F^5U, but the TLC conditions did not resolve the hydrated F^5U isomers from each other. The nuclease P1/alkaline phosphatase digestion

[1]Abbreviations: Ψ, pseudouridine; F^5U, 5-fluorouridine; [F^5U]tRNA, the *in vitro* transcript of *Escherichia coli* tRNA^{Phe} containing F^5U in place of U; TSL, T-arm stem−loop; [F^5U]TSL, TSL containing F^5U in place of the U isomerized by TruB; ASL, anticodon stem−loop; [F^5U]ASL, ASL containing F^5U in place of the U isomerized by RluA; nESI, nanoelectrospray ionization; MALDI, matrix-assisted laser desorption ionization; TOF, time of flight; TruB products, the products of F^5U resulting from action of TruB on [F^5U]TSL; RluA products, the products of F^5U resulting from the action of RluA on [F^5U]ASL; TruA products, the products of F^5U resulting from the action of TruA on [F^5U]tRNA.
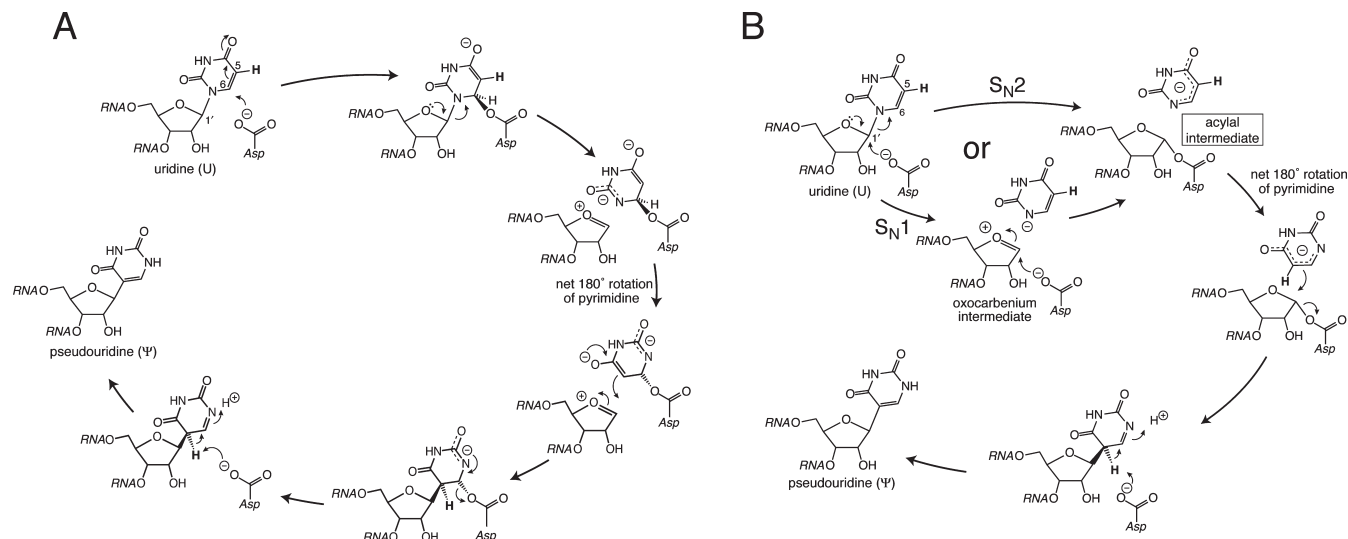
FIGURE 1: The two mechanisms proposed for Ψ synthases. (A) The catalytic Asp is a nucleophile in a Michael addition to the pyrimidine ring. (B) The catalytic Asp is a nucleophile in the formation of an acyl intermediate. The stereochemistry at C5 and C6 in intermediates and products reflects that observed in the cocrystal of TruB and [F^{5}U]TSL (*8*). The depiction of the catalytic Asp as the general base for the final deprotonation rests on the interpretation of the pH dependence of the TruB reaction (*20*) and differs from conclusions drawn by Phannacet et al. (*15*), as has previously been discussed in some detail (*12*).
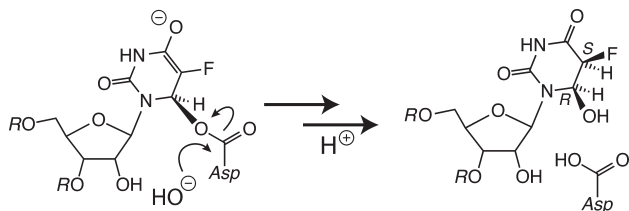

FIGURE 2: The hydrolysis of the initial Michael adduct between F^{5}U in RNA and the catalytic Asp proposed by Gu et al. (*7*) followed by implicit protonation of C5.

products were also examined by reverse-phase HPLC, which cleanly resolves the two isomers of photohydrated F^{5}U. As judged by comparison of the UV trace with the tritium content of collected fractions, the nucleoside product of F^{5}U coeluted from the reverse-phase HPLC column with one isomer of authentic hydrated F^{5}U. From these results, Santi and co-workers (*7*) reasonably concluded that TruA operates via the mechanism involving Michael addition of the active site Asp to the pyrimidine ring, with the enzyme "getting stuck" at the first ester intermediate, which neatly explains the irreversible inhibition and covalent adduct. When heated, the intermediate underwent ester hydrolysis to generate hydrated F^{5}U (Figure 2).

This general scenario seemed to be supported by the cocrystal structure of the Ψ synthase TruB with an RNA stem—loop corresponding to its natural substrate but containing F^{5}U in place of the isomerized U (*8*). A covalent adduct was not observed, but the product of F^{5}U was hydrated; surprisingly, that product was also rearranged to the *C*-glycoside as U is rearranged to generate Ψ. The crystallographers reasonably concluded that the enzyme proceeded beyond the initial ester intermediate to the *C*-glycoside, the ester group of which underwent slow hydrolysis during crystal growth and data acquisition (*8*). Subsequent examination of TruB and its [F^{5}U] stem—loop revealed evidence for neither potent inhibition nor the formation of a covalent adduct but instead indicated that TruB handles the F^{5}U as a substrate and converts it into two products that are partially resolvable by reverse-phase HPLC (*9*). MALDI-MS analysis of the oligonucleotides resulting from the digestion of product

stem—loops with RNase T$_1$ (which cuts after G) demonstrated that the product of F^{5}U was hydrated. Labeling studies with [^{18}O]water definitively showed, however, that the hydrated products did not arise from ester hydrolysis (*10*). Although disallowing the reasonable conclusion that hydrated products indicated that the Michael mechanism was followed, the labeling results were consistent with both proposed mechanisms.

A similar suite of experiments with the Ψ synthase RluA revealed that it behaved similarly to TruA in its stoichiometric irreversible inhibition by [F^{5}U]RNA with apparent covalent adduct formation (*9*). A cocrystal of RluA and [F^{5}U]RNA, however, did not reveal the expected covalent linkage, but the adduct was shown to be sensitive to X-irradiation and so likely decomposed in the crystal (*11*). After heat disruption of the adduct and S1 nuclease/phosphatase digestion, HPLC analysis revealed a single product peak of F^{5}U from the action of RluA that did not coelute with either product of F^{5}U from the action of TruB, and MALDI-MS analysis of the oligonucleotides resulting from the RNase T$_1$ digestion of product stem—loop confirmed that RluA causes the hydration of F^{5}U (*12*). ^{18}O labeling studies confirmed that hydration did not result from ester hydrolysis (*12*).

TruA, TruB, and RluA each belong to different Ψ synthase families, and even though they share the same overall fold and active site architecture, it remained possible that interfamilial variation could lead to different outcomes from the same suite of experiments. The labeling studies were extended to TruA to examine whether or not ester hydrolysis occurs during the hydration of F^{5}U. Further characterization of the products of F^{5}U from the action of each enzyme was undertaken to ascertain whether or not Ψ synthases of different families handle F^{5}U differently. In particular, the behavior of the nuclease P1/alkaline phosphatase digestion products on reverse-phase HPLC was examined to check the assignment of the product of F^{5}U from TruA action as the hydrate of the parent nucleoside (with the C—N glycosidic bond intact as opposed to the C—C glycosidic bond observed in the TruB cocrystal) and the apparent formation of two products of F^{5}U by TruB action as opposed to a single different product by RluA action.

MATERIALS AND METHODS

General. Chemicals were purchased from Fisher Scientific (Pittsburgh, PA) or its Acros Organics division (Pittsburgh, PA) unless otherwise noted. Alkaline phosphatase and S1 nuclease[2] were purchased from Promega (Madison, WI). Radiant Red RNA stain was purchased from Bio-Rad (Hercules, CA).[3] SYBR Gold nucleic stain was obtained from Invitrogen (Carlsbad, CA). T7 RNA polymerase was purchased from Epicentre Biotechnologies (Madison, WI), and MEGAshortscript T7 transcription kits were purchased from Applied Biosystems (Foster City, CA). Electroelution of RNA from polyacrylamide gels was accomplished using an Elutrap device (Whatman Inc., Piscataway, NJ) following the manufacturer's protocol. "UV shadowing" was used to locate RNA-containing gel bands; the gel was laid on plastic wrap over a silica TLC plate impregnated with a fluorescent indicator, and the gel was illuminated with a hand-held UV lamp, resulting in a "shadow" on the plate beneath the RNA-containing band (because the RNA absorbs UV light). All experiments were performed with water ($\geq$18.2 MΩ) from a Milli-Q Synthesis system (Millipore, Billerica, MA). Reaction buffer is 50 mM HEPES, pH 7.5, containing ammonium chloride (100 mM), dithiothreitol (5 mM), and EDTA (1 mM).

HPLC. HPLC was performed either on an Agilent 1100 system composed of a binary pump, micro vacuum degasser, variable-wavelength detector, and thermostated autosampler with an extended volume upgrade kit run by ChemStation software (Agilent Technologies, Santa Clara, CA) or on a Beckman System Gold system composed of a 125 gradient pump module, 508 autosampler, and 168 photodiode array detector run by 32Karat XP software (Beckman Coulter, Fullerton, CA). Analytical runs of S1 nuclease/alkaline phosphatase digestion products were performed as described previously (*12*). Preparative runs of the products of F[5]U are described in detail below.

NMR. NMR spectra were acquired using INOVA 500 NMR spectrometers (Varian Inc., Palo Alto, CA) equipped with either a 5 mm inverse HFX z-PFG probe (Wang NMR, Inc., Palo Alto, CA) or a 5 mm direct broad-band probe (Varian). Spectra were processed with VNMRJ v2.1c (Varian) or MestReC v4.9.9.6 (Mestrelab Research, Santiago de Compostela, Spain).

RNA Oligonucleotides and Their Digestion. Synthetic RNA oligonucleotides with 2′-ACE protecting groups were purchased from Dharmacon (Boulder, CO), deprotected according to the manufacturer's protocol, and desalted as previously described (*12*). The RNA oligonucleotide corresponding to the *anticodon stem−loop* of *E. coli* tRNA^Phe with F[5]U in place of the isomerized U, [F[5]U]ASL, is GGGGAF[5]UUGAAAAUCCCC. The RNA oligonucleotide corresponding to the *T*-arm *stem−loop* of *Saccharomyces cerevisiae* tRNA^Phe with F[5]U in place of the isomerized U, [F[5]U]TSL, is CUGUGUF[5]UCGAUCCACAG.

Digestion of RNA with S1 nuclease and alkaline phosphatase was achieved as described previously with the latter enzyme added directly to the sample in nuclease buffer (*12*). For large-scale sample preparation, glycerol was removed from S1 nuclease and alkaline phosphatase before their use by exchanging them

into the manufacturer's S1 nuclease buffer by dialysis, ultrafiltration, or spin size-exclusion chromatography.

Synthesis of F[5]U Derivatives. 5-Fluorouridine (Acros), F[5]U, was phosphorylated by the method of Darlix et al. (*13*) to afford F[5]UMP, which was converted to F[5]UTP enzymatically using the method of Tolbert and Williamson (*14*). F[5]U was photohydrated to the 5*S*,6*R* and 5*R*,6*S* isomers of 5,6-dihydro-6-hydroxy-5-fluorouridine according to the method of Gu et al. (*7*). Detailed procedures and characterization data can be found in the Supporting Information.

In Vitro Transcription of [F[5]U]tRNA. The *in vitro* transcription of *E. coli* tRNA^Phe was performed either as described previously (*3*) with the substitution of F[5]UTP (4 mM) for UTP or using MEGAshortscript T7 transcription kits according to the manufacturer's protocol with F[5]UTP (2.25 mM) substituted for UTP. The resulting crude [F[5]U]tRNA was purified by urea−PAGE (5% gel, 30 min, 160 V, or 6% gel, 80 min, 150 V; both at room temperature); the band was located by UV shadowing for excision and subsequent electroelution. All batches of [F[5]U]-tRNA were characterized by MALDI-MS after digestion with RNase A and/or RNase T$_1$. Concentration was determined by A_{260} using an extinction coefficient of 5×10^5 M^{-1} cm^{-1}, and aliquots were quick-frozen and stored at −80 °C. Before use, aliquots were quick-thawed, incubated at 100 °C for 5 min, and then snap-cooled.

Overexpression and Purification of Ψ Synthases. TruB was overexpressed, purified, and stored as previously described (*3*) with an additional polishing step over a column (4.6 × 100 mm) of POROS HQ anion-exchange resin (Applied Biosystems), eluting with a gradient (0−1.5 M) of sodium chloride in 50 mM Tris·HCl buffer, pH 8.5. RluA was overexpressed, purified, and stored as previously described (*11*).

TruA was overexpressed in *E. coli* BLR(DE3) pLysS cells (Novagen, Madison, WI) from a plasmid (pBH500) with *truA* inserted in-frame into pET15b (Novagen) at the *Nde*I site to cause expression of TruA with an N-terminal His$_6$ tag immediately preceding Met-1 of the native enzyme. The cells were lysed, and the cell extract was treated and subjected to chromatography over a column of Ni-NTA resin (Novagen) as described previously for TruB and RluA (*3*). The TruA-bearing fractions were combined and dialyzed against 50 mM potassium phosphate buffer, pH 6.5, and then subjected to chromatography over a column (4.6 × 100 mm) of POROS HS cation-exchange resin (Applied Biosystems), eluting with a gradient (0−1.5 M) of potassium chloride in the same buffer. TruA-bearing fractions were tested for RNase activity, and clean fractions were combined and dialyzed into 20 mM Tris·HCl buffer, pH 7.5, containing EDTA (0.1 mM), sodium chloride (300 mM), β-mercaptoethanol (5 mM), and glycerol (10% v/v). TruA was typically stored at 4 °C and used within 1 month; for longer storage at −20 °C, glycerol was added to 50% (v/v). An extinction coefficient at 280 nm of 41840 M^{-1} cm^{-1} was calculated using A_{280} and the protein concentration determined by biuret assay using bovine serum albumin as the standard, as previously described (*3*).

Formation and Denaturing Gel Analysis of the Tru-A-[F[5]U]RNA Adduct. TruA (8−40 μM) and [F[5]U]tRNA (1−16 μM) were incubated for 5 min to 48 h at either 20 or 37 °C in 20 mM Tris·HCl buffer, pH 8.0, containing ammonium chloride (100 mM) and dithiothreitol (2 mM) (*2*). The various concentrations of protein and RNA in the numerous experiments ranged from a stoichiometry of 10:1 to 2:5 TruA:[F[5]U]tRNA. To measure the extent of adduct formation, aliquots of the incubations

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[2] S1 nuclease has the same enzymatic activity and specificity as nuclease P1, which we used previously (*21*) to get digestion products that displayed identical HPLC behavior as the ones reported here with S1 nuclease. The change in nuclease, therefore, does not account for the difference in products reported by Santi and co-workers (*7*) and Huang and co-workers (*15*).

[3] Radiant Red ceased to be commercially available during the course of this work.

were subjected to SDS−PAGE (10% gel) or urea−PAGE (5% or 6% gel; 8 M urea) both with and without prior incubation of the samples at 100 °C for 5 min. Gels were stained first with Radiant Red or SYBR Gold to detect RNA[3] and then with Coomassie Blue to detect protein. Complete adduction was very rarely observed. Suspecting that in-gel decomposition might be reducing the apparent yield of adduct, minigels (7 cm × 8 or 10 cm × 10 cm) were run at 4 °C and lower voltage (60 versus 160 V), or larger format (16 cm ×16 cm) gels were run in a chamber (Hoefer SE 600; GE Healthcare) with a heat exchanger connected to a circulating bath to maintain constant temperature over the gel throughout the run. These measures only modestly improved the yield. In the end, the best results were obtained with an excess of TruA over [F⁵U]RNA (5:2), which typically led to 65−70% of (limiting) RNA found in the adduct based on urea−PAGE analysis.

[18]*O Labeling Studies.* To determine whether oxygen from solvent was incorporated into the active site Asp-60 of TruA or into the hydrated product of F^5U, the TruA-[F^5U]tRNA adduct was formed in buffer containing 50% [^{18}O]water, purified, and heat-disrupted, followed by the separate MALDI-MS analysis of protein and RNA after digestion with trypsin and RNase T_1, respectively, to determine the location of the isotopic label. The adduct between TruA and [F^5U]tRNA was formed by incubating [F^5U]tRNA (10 μM) for 5 h at room temperature with TruA (40 μM) in 20 mM Tris·HCl buffer, pH 8.0, containing ammonium chloride (100 mM) and dithiothreitol (2 mM) (*2*). Adduction was confirmed by SDS−PAGE analysis of a small aliquot. The bulk of the reaction mixture was diluted with an equal volume of neat formamide and subjected to urea−PAGE. The adduct band was located by UV shadowing and excised, and the adduct was electroeluted and concentrated in a Microcon-3 ultrafiltration device (Millipore). The sample was then incubated for 5 min at 100 °C, and precipitated protein was pelleted by centrifugation. The supernatant was washed with phenol/chloroform and the RNA precipitated with ethanol. The digestion and MALDI-MS analysis of the TruA and RNA from the disrupted adduct followed the procedure described in detail for the similar study of the adduct between RluA and [F^5U]RNA (*12*) except that 6-aza-2-thiothymine was used as the matrix for MALDI-MS of oligonucleotides; the procedure includes the addition of alkaline phosphatase after RNase to remove 3′-phospho groups, which themselves incorporate an oxygen atom from solvent during the digestion. For comparison, the adduct was formed separately in both labeled and unlabeled buffer, and after gel purification, heat disruption of the adduct was conducted in both labeled and unlabeled buffer. All four combinations were covered: both formation and disruption in unlabeled buffer; formation in unlabeled and disruption in labeled buffer; formation in labeled and disruption in unlabeled buffer; both formation and disruption in labeled buffer.

Large-Scale Preparation of the Products of F⁵U from the Action of TruB. TruB (10 μM) and [F^5U]TSL (100 μM) were incubated in reaction buffer (400 μL). After 3 h at 37 °C, an aliquot (3.5 μL) was removed and subjected to digestion and HPLC analysis in order to verify that the reaction was complete. The protein was removed from the aqueous phase by vortex mixing with an equal volume of phenol saturated with TE buffer, pH 4.3, and then with an equal volume of chloroform/isoamyl alcohol (24:1). The RNA in the aqueous layer was precipitated by adding first sodium acetate buffer, pH 4.6 (to 300 mM), and then

an equal volume of cold absolute ethanol (−20 °C). After incubation for at least 3 h at −20 °C, the precipitate was collected by cold centrifugation (30 min, 13100*g*). The pellet was gently washed twice with cold aqueous ethanol (70% v/v; −20 °C) and air-dried. The RNA pellet was dissolved in S1 nuclease buffer and digested with S1 nuclease (450 units) and alkaline phosphatase (9 units).

The resulting nucleoside products were purified by reverse-phase HPLC over a preparatory Ultrasphere C_{18} column (5 μm, 10 × 250 mm; Beckman-Coulter) with an acetonitrile gradient by use of the following program (the first number is the percentage of aqueous acetonitrile (40% v/v); the second number is the elapsed time in minutes): 0, 0; 0, 3; 5, 8; 15, 18; 15, 23; 30, 26; 50, 27.5; 50, 29; 100, 30; 100, 31; 0, 32; 0, 39; 0, 40. All gradient steps were linear. Fractions (1.5 mL) were subjected to analytical HPLC to locate the products of F^5U. Product-bearing fractions were combined, taken to dryness *in vacuo*, redissolved in 50 mM sodium phosphate buffer in D_2O (500 μL), pD 7.9, containing sodium 4,4-dimethyl-4-silapentane-1-sulfonate-d_6 (0.12 mM; kindly provided by T. Fan, University of Louisville), and subjected to NMR and nESI-MS analysis.

Large-Scale Preparation of the Products of F⁵U from the Action of RluA. RluA (200 μM) and [F^5U]ASL (150 μM) were incubated for 3 h at 37 °C in standard assay buffer (1.6 mL), with the completion of reaction verified by SDS−PAGE gel shift analysis. The [F^5U]ASL−RluA adduct was concentrated by using an Amicon Ultra-10 centrifugal ultrafiltration device (Millipore), and to remove unreacted RluA and [F^5U]ASL, the crude adduct sample was purified as previously described (*11*) with slight variation. The adduct was subjected to anion-exchange chromatography over a column of POROS HQ resin (Applied Biosystems). Elution was achieved with a linear gradient of sodium chloride (0−1 M) in 30 mM Tris·HCl buffer, pH 8.5, containing EDTA (1 mM) and DTT (1 mM). Adduct-bearing fractions were identified by SDS−PAGE analysis, combined, and concentrated in an Amicon Ultra-10 ultrafiltration device. To achieve heat disruption of the adduct, the sample was incubated for 5 min at 100 °C in a dry heating block. Heat-precipitated RluA was pelleted by centrifugation (10 min, 18000*g*), and the supernatant was spin-filtered (0.22 μm, cellulose acetate; Corning, Upton, NY).

The modified [F^5U]ASL was simultaneously digested and dephosphorylated by the addition of glycerol-free S1 nuclease (450 units) and calf intestine alkaline phosphatase (9 units). After overnight at 37 °C, the reaction was judged complete from the reverse-phase HPLC analysis of an aliquot (2.5 μL). The product of F^5U was then purified by reverse-phase HPLC over a preparatory C_{18} column as described above for the products of F^5U from TruB action. Product-bearing fractions were combined, taken to dryness *in vacuo*, and redissolved in 50 mM sodium phosphate buffer in D_2O (500 μL), pD 7.9, containing sodium 4,4-dimethyl-4-silapentane-1-sulfonate-d_6 (0.12 mM) for analysis by NMR and nESI-MS.

Mass Spectrometry. Matrix-assisted laser desorption ionization spectrometry, MALDI-MS, was performed on either a Ominflex (Bruker Daltonics, Inc., Billerica, MA) or a Voyager DE-PRO (Applied Biosystems) MALDI-TOF mass spectrometer as described previously (*12*). Nanoelectrospray ionization mass spectrometry, nESI-MS, was performed using an LTQ-FT mass spectrometer (Thermo Scientific, Waltham, MA) equipped with a TriVersa NanoMate direct infusion nano-ESI ion source (Advion BioSystems, Ithaca, NY). Aliquots (0.25−0.5 μL) of the

NMR samples of the products of F^5U from the action of TruB and RluA were diluted into water (25 μL), to which methanol (25 μL; Optima* LC/MS grade; Fisher) was then added. The mass spectra were acquired after at least 10 min at room temperature. The products of F^5U from the action of TruA were analyzed similarly by dilution of the collected HPLC peaks with water and methanol; the quality of the mass spectrum was lower than for the other two cases, presumably because of ion suppression by buffer in the HPLC eluate. To increase sensitivity, a narrow range (± 3 m/z) around the ion of interest was isolated in the linear ion trap and then injected into the FT-ITCR to acquire high-resolution and high-accuracy spectra from 100 to 1000 m/z. The selected ranges (259−265, 277−83, 338−344, 356−362, 563−569, and 581−587) encompassed the candidate products (rearranged F^5U, hydrated F^5U, the monophosphate of F^5U, the monophosphate of hydrated F^5U, the dinucleotide of rearranged F^5U and the following C, and the dinucleotide of hydrated F^5U and the following C).

RESULTS

Synthesis of F^5UTP and $[F^5U]tRNA$. F^5UTP was obtained by the chemical phosphorylation of F^5U, isolation of the resulting F^5UMP, its subsequent enzymatic conversion to F^5UTP, and purification by anion-exchange chromatography. The F^5UTP was used in place of UTP during the *in vitro* transcription of *E. coli* tRNA[Phe].

Adduct Formation. TruA and $[F^5U]tRNA$ were incubated together under many permutations of relative concentration, absolute concentration, temperature, and time, but the limiting partner was very rarely driven completely into the adduct as judged by denaturing gel analysis (Figure 3, which shows a rare near quantitative adduction). The routine yield of adduct was 65−70%. Running the gels at lower temperature did not significantly improve the apparent yield of adduct, strongly suggesting that decomposition in the gel does not account for the apparently incomplete reaction of limiting RNA or limiting enzyme. Huang et al. (*2*) reported complete adduction of $[F^5U]tRNA$ after overnight incubation with 25-fold excess TruA; their yield of 75% after 5 h is consistent with our typical results. Incomplete adduction does not compromise the results reported here since the $[F^5U]tRNA$−TruA adduct was gel-purified before subjecting it to the characterization methods described below.

Characterization of the Adduct Components by MALDI-MS. The gel-purified adduct was disrupted by heating; the denatured TruA was pelleted by centrifugation, and the RNA was ethanol-precipitated from the removed supernatant. The pelleted TruA was digested with trypsin, and the precipitated $[F^5U]tRNA$ was digested with RNase T_1 (cuts after G). The resulting oligopeptides and oligonucleotides were analyzed by MALDI-MS as described previously (*9, 10, 12*). The tryptic peptides covered 41% of the TruA sequence, including [59]TDAGVH-GTGQVVHFETTALR[78] (2096.05 $m/z_{predicted}$; 2095.98 $m/z_{observed}$), which contains the active site Asp-60. Digestion with RNase T_1 and alkaline phosphatase produced the expected oligonucleotides, which were compared to the oligonucleotides from digestion of $[F^5U]tRNA$ not incubated with TruA. The oligonucleotide containing F^5U39 ([35]AAAA(F^5U)CCCCG[44]; 3142.47 $m/z_{predicted}$; 3142.50 $m/z_{observed}$) shifted +18.05 m/z after adduct formation/disruption, clearly indicating that F^5U39 suffered hydration ([35]AAAA(F^5U-H_2O)CCCCG[44]; 3160.46 $m/z_{predicted}$; 3160.55 $m/z_{observed}$).
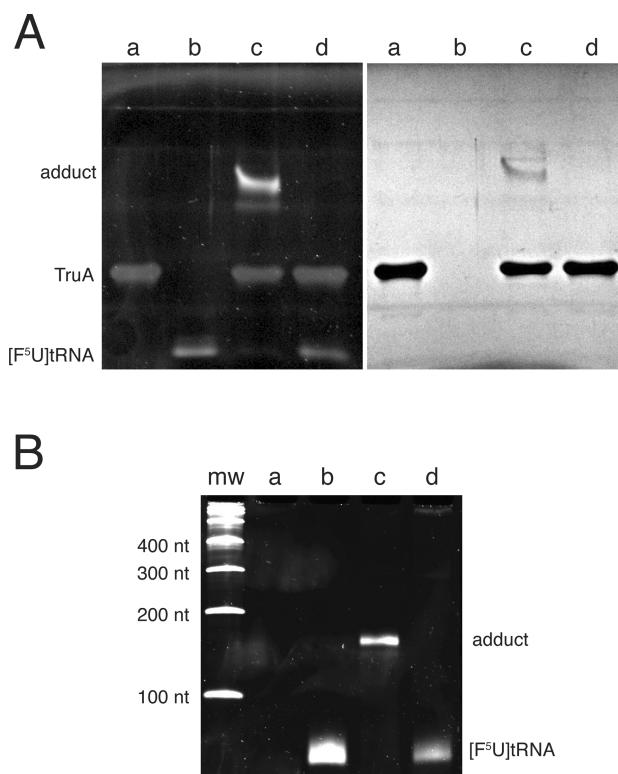

FIGURE 3: Gel shift results implying a covalent adduct between TruA and $[F^5U]tRNA$. Adduct was formed by incubating TruA (20 μM) and $[F^5U]RNA$ (10 μM) at 20 °C for 20 h. (A) SDS−PAGE stained for RNA with SYBR Gold (left) and then for protein with Coomassie Blue (right). a, TruA; b, $[F^5U]tRNA$; c, adduct; d, heat-disrupted adduct. RNA in the band consistently appears to decrease the efficiency of Coomassie Blue staining, leading to an adduct band (54.6 kDa $MW_{measured}$; 57.0 kDa $MW_{predicted}$) that is lighter than expected based on the staining of an equal amount of loaded TruA (31.7 kDa $MW_{measured}$; 32.5 kDa $MW_{predicted}$) and $[F^5U]tRNA$ (lowest band). The faint staining of protein by SYBR Gold is apparent. The band that runs at slightly lower $MW_{apparent}$ than the main adduct band is presumed to be a conformational isomer or a product of limited hydrolysis of TruA or $[F^5U]tRNA$; the low abundance of the band (< 10% of the total adduct) precluded further characterization. (B) Urea−PAGE stained for RNA with SYBR Gold. mw, RNA markers; a, TruA; b, $[F^5U]tRNA$; c, adduct; d, heat-disrupted adduct. The adduct band (167 nt measured) runs slower than $[F^5U]tRNA$ (70 nt measured; 76 nt predicted). Coomassie Blue faintly stains the adduct and reveals that TruA mostly collects at the well bottom and enters the gel as a diffuse streak (data not shown).

^{18}O Labeling. To determine the source of oxygen added to F^5U during adduct formation/disruption, the adduct was formed by incubating $[F^5U]RNA$ with TruA in buffer that contained 50% $[^{18}O]$water. The gel purification was run entirely in unlabeled buffer, and the purified adduct was put back into buffer containing 50% $[^{18}O]$water before heat disruption. The precipitated TruA and product $[F^5U]tRNA$ were separated, digested, and analyzed by MALDI-MS as was the unlabeled sample.

The mass spectra (Figure 4) clearly indicate that ^{18}O ends up not in Asp-60 but in F^5U39. A control in which the trypsinolysis was performed in buffer containing 50% $[^{18}O]$ water (so that 50% of the newly generated peptide C-terminal carboxylates will contain ^{18}O rather than ^{16}O) confirms that ^{18}O in Asp-60 would be immediately detectable (data not shown). Other controls described previously (*10, 12*) to test for exchange of oxygen between solvent and carboxylate groups demonstrated that no such exchange occurs in TruA under any of the experimental conditions. The complementary analysis of the product oligonucleotides from
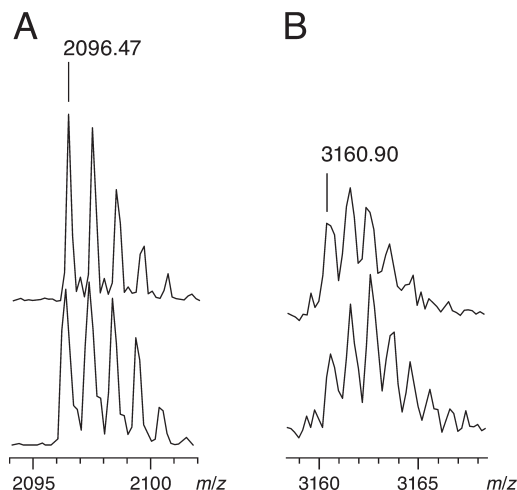
FIGURE 4: Partial MALDI mass spectra showing incorporation of [18]O from solvent into RNA rather than protein. (A) The oligopeptide ([59]TDAGVHGTGQVVHFETTALR[78]; 2096.05 $m/z_{predicted}$) containing the catalytic Asp-60 after TruA from heat-disrupted adduct was subjected to trypsinolysis: upper trace, adduct was formed and disrupted in unlabeled buffer; lower trace, adduct was formed and disrupted in buffer containing 50% [^{18}O]water. (B) The oligonucleotide ([35]AAAA(F[5]U-H$_2$O)CCCG[43]; 3160.46 $m/z_{predicted}$) containing the reactive F[5]U after [F[5]U]tRNA from heat-disrupted adduct was subjected to digestion with RNase T$_1$: upper trace, adduct was formed and disrupted in unlabeled buffer; lower trace, adduct was formed and disrupted in buffer containing 50% [^{18}O]water.

[F[5]U]tRNA confirms that [18]O is incorporated directly into F[5]U (Figure 4), and controls (*10, 12*) demonstrate that the incorporated oxygen does not exchange with solvent after the hydration event.

To probe whether hydration of F[5]U occurs during adduct formation or disruption, the analysis was repeated with adduct prepared in labeled buffer and disrupted in unlabeled buffer and *vice versa*. As expected, [18]O was not incorporated into Asp-60 in either case (data not shown). Informatively, the isotopic content of the product oligonucleotides containing F[5]U39 matched the isotopic content of the buffer *in which heat disruption* was performed. In other words, adduct prepared in unlabeled buffer and disrupted in buffer containing [^{18}O]water produced an identical mass spectrum to adduct both prepared and disrupted in labeled buffer (data not shown). Conversely, adduct prepared in labeled but disrupted in unlabeled buffer yielded a mass spectrum identical to the case in which [^{18}O]water was never present (data not shown).

NMR Analysis of F[5]U Products. The appearance of two partially resolved peaks in the C$_{18}$ HPLC analysis of the RNA digestion products after incubation of [F[5]U]TSL with TruB indicates that the enzyme converts F[5]U two products (*10*), but a similar analysis of [F[5]U]ASL after incubation with RluA gives a single product peak (*12*). To start the definitive chemical identification of these distinct products, they were prepared and purified on a scale sufficient to allow the acquisition of their NMR spectra (Figure 5). First, [F[5]U]TSL was incubated with
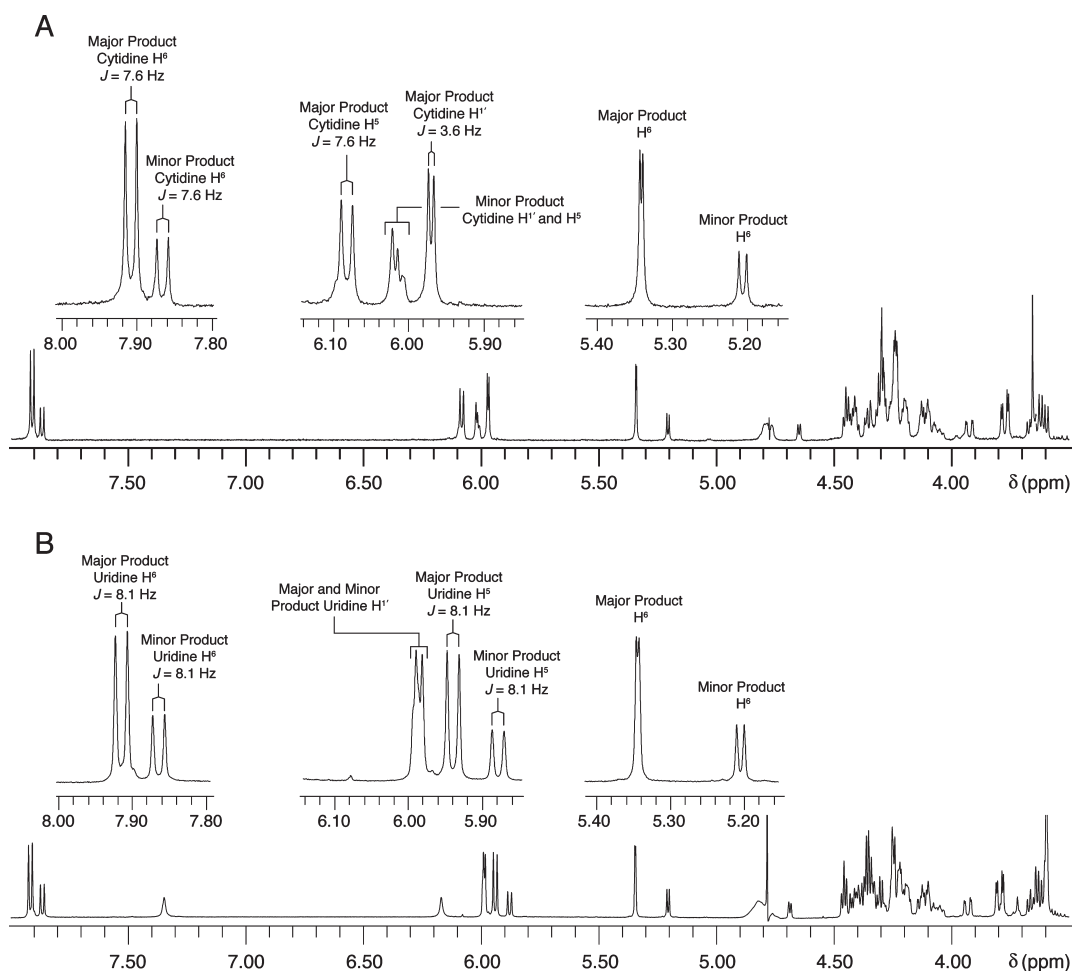

FIGURE 5: ^{1}H NMR spectra of the products of F[5]U from Ψ synthase action. (A) The TruB products. (B) The RluA products. The appearance of cytidine and uridine resonances in the TruB and RluA products, respectively, indicates that digestion by S1 nuclease produced dinucleotides between the product of F[5]U and the nucleoside that follows it in the RNA. Both samples consist of a major and a minor product, accounting for the appearance of two sets of cytidine and uridine resonances (pyrimidine protons, left insets).

TruB (0.1 equiv); after HPLC analysis revealed that reaction was complete, the protein was extracted with phenol/chloroform. The RNA was ethanol-precipitated and digested with S1 nuclease (nonsequence specific; generates nucleoside 5′-monophosphates) and alkaline phosphatase. The ^{1}H NMR spectrum (Figure 5, panel A) of the products of F^5U from the action of TruB ("TruB products" for ease of reference) unexpectedly reveals the presence of twice the expected ribose protons (3.5−4.6 ppm) and of a pair of cytidine protons (identified by chemical shift and $J_{H^5-H^6}$ values) in the same ratio as the integration of the major and minor product peaks in the HPLC trace. This result immediately suggested that the major and minor products were both *di*nucleotides of the hydrated F^5U products and the C that follows it in [F^5U]TSL rather than the expected *mono*nucleosides. The ^{31}P NMR spectrum (data not shown) consisted of two resonances in the same ratio as the cytidine peaks in the ^{1}H NMR spectrum and the integration of the HPLC trace, indicating that both products contained a bridging phospho group.

To obtain a sufficient quantity of the product of F^5U from the action of RluA on [F^5U]ASL ("RluA product"), the adduct formed by incubation of the two was prepared on a large scale and purified by anion-exchange chromatography. After heat disruption, the precipitated protein was pelleted by centrifugation, and the RNA was digested with S1 nuclease and alkaline phosphatase. The nucleoside products were separated by preparative HPLC. Although the "RluA product" was contained in a single peak, NMR analysis (^{1}H, Figure 5, panel B; ^{31}P, not shown) clearly revealed that two dinucleotide products were present in roughly the same ratio as seen between the major and minor TruB products but with the hydrated F^5U products followed by U (rather than C) as expected from the sequence of [F^5U]ASL.

Mass Spectrometric Analysis of F^5U Products. An earlier report of the mass spectra of the TruB products stated that both were hydrated *mono*nucleosides, but the mass spectrum itself was not included (*15*). Given that the NMR results indicated *di*nucleotide products, resolution of this apparent discrepancy was imperative. A small portion of the NMR samples of the TruB products and the RluA products was therefore subjected to nESI-MS analysis (Figure 6). The observed molecular ions matched dinucleotides of hydrated F^5U and C in the case of the TruB products and of hydrated F^5U and U in the case of the RluA products.[4] The mass spectra lacked peaks corresponding to either the nucleoside or nucleoside monophosphate of hydrated F^5U. Furthermore, the mass spectra contained no peaks corresponding to unhydrated F^5U, either alone or as a dinucleotide with the following residue, which indicates that all products of F^5U are hydrated in agreement with the MALDI-MS data from digestion to oligonucleotides. The mass difference between the TruB and RluA products corresponds to the 1 Da difference between C and U, indicating that the major and minor products from the action of both enzymes are composed of isomers of hydrated F^5U.

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[4]The mass spectra cannot, of course, distinguish between hydrated F^5U and its skeletal or stereochemical isomers. The isotopic equilibration of the deuterium on the products in the NMR samples with the vast excess of unlabeled water (and methanol) is expected to be very fast and should be easily achieved in the time between dilution and acquisition of the mass spectra. In support of full equilibration, no change in the monoisotopic masses was observed during replicate runs of the samples (data not shown), and the products of TruB action have the same monoisotopic mass as those from the action of TruA, which were never exposed to D_2O.
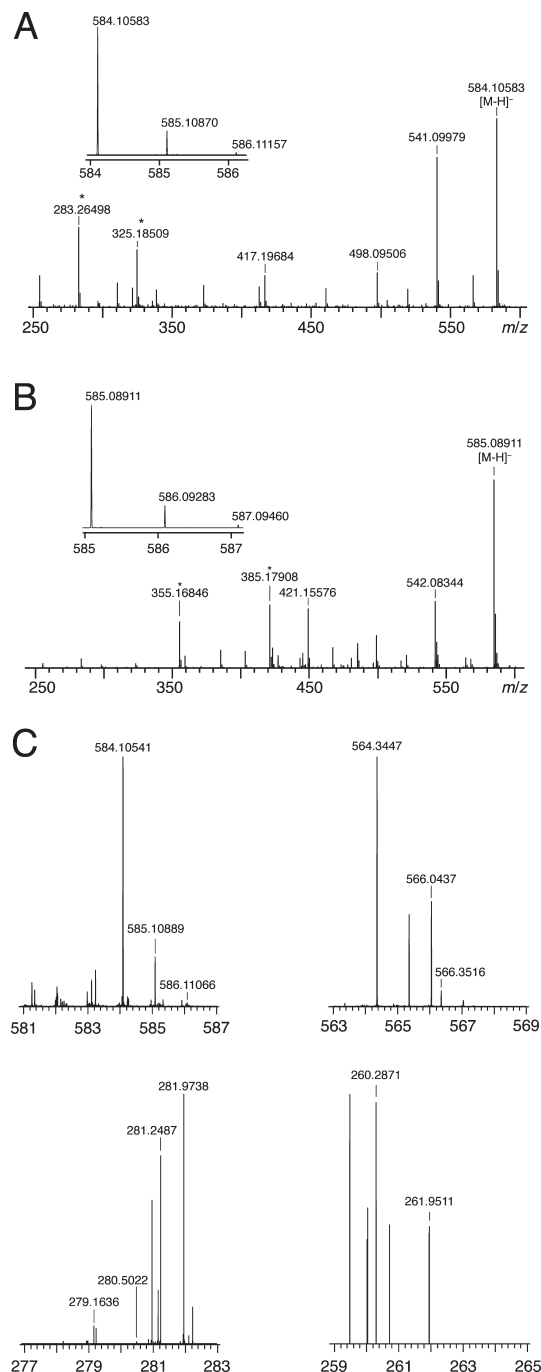

FIGURE 6: nESI-MS analysis of the products of F^5U. (A) The TruB products (584.10414 $m/z_{predicted}$ for the dinucleotide of hydrated, rearranged F^5U and C; 584.10583 $m/z_{observed}$). (B) The RluA products (585.08816 $m/z_{predicted}$ for the dinucleotide of hydrated, rearranged F^5U and U; 585.08911 $m/z_{observed}$). (C) The TruA products; mass spectra are from a 6 m/z range of ions spanning candidate products that were isolated in a linear ion trap: 581−587 (the dinucleotide of hydrated, rearranged F^5U and C, 584.10414 $m/z_{predicted}$; 584.10541 $m/z_{observed}$), 563−569 (the dinucleotide of rearranged F^5U and C, 566.0936 $m/z_{predicted}$; not observed), 277−283 (hydrated F^5U, 280.0707 $m/z_{predicted}$; not observed), 259−265 (rearranged F^5U, 262.0601 $m/z_{predicted}$; not observed); ranges acquired but not shown: 356−362 (hydrated F^5UMP, 359.0292 $m/z_{predicted}$; not observed) and 338−344 (F^5UMP, 341.0186 $m/z_{predicted}$; not observed). Measured m/z values have an uncertainty of ±2.5 ppm or ±0.0014 for 566 m/z and ±0.0007 for 262 m/z; no peaks match candidate products within this tolerance other than the dinucleotide of hydrated, rearranged F^5U and C. Asterisks denote background peaks present in dummy runs.
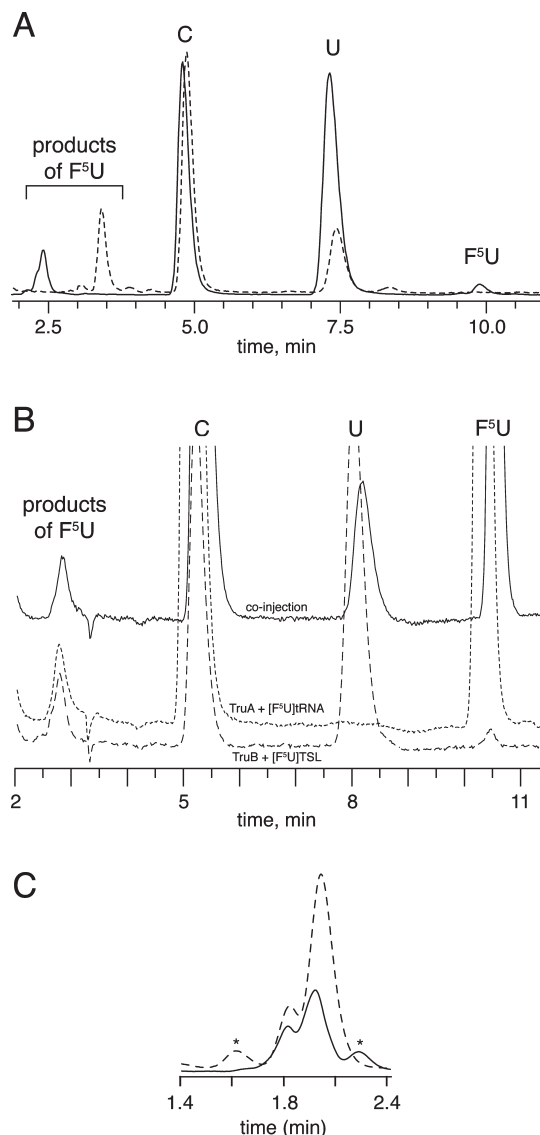
Table 1: Quantitation of Peaks from the HPLC Traces of Digested $[F^5U]RNA^a$

	C	U	F^5U^b	G	A	products
$[F^5U]$TSL	**5.2**	3.9	0.67	4.1	5.0	0.0
$[F^5U]$TSL + TruB	**4.3**	3.8	0.10	4.0	5.0	0.7
$[F^5U]$ASL	4.0	**2.0**	0.72	5.0	5.0	0.0
$[F^5U]$ASL + RluA	4.3	**1.3**	0.10	5.2	5.0	0.6
$[F^5U]$tRNA	**20.8**		11.1	22.3	15.0	0.0
$[F^5U]$tRNA + TruA	**20.4**		10.8	23.1	15.0	0.67
$[F^5U]$ASL(U → C)^c	**4.7**	1.0	0.7	5.0	4.9^d	0
$[F^5U]$ASL(U → C)^c + RluA	**4.0**	0.9	0.2	5.0	5.0^d	0.5

^{a}Relative integration (A_{260}) was calculated by adjusting the arbitrary integration units using ε_{260} for each species; for products, ε_{260} of the nucleotide following F^5U (either C or U) in the RNA sequence was used. Adenosine was used to normalize all values (except as noted; see footnote d), which have an error of ±0.2. Bold type indicates the "missing" nucleoside integration (beyond the expected decrease of F^5U) after incubation with the Ψ synthase that is accounted for by dinucleotide products. ^{b}Quantitation of F^5U is difficult even with great care because it is highly sensitive to pH variations in the injected samples, presumably due to its lower pK_a value relative to the other nucleosides. $^c[F^5U]$ASL in which the U following the F^5U is replaced with C. ^{d}The lot of S1 nuclease used for this set of experiments was contaminated by adenosine deaminase, which led to the substantial hydrolysis of adenosine to inosine. The sum of adenosine and inosine is reported in the "A" column, and guanosine was used to normalize the values.

FIGURE 7: HPLC analysis of the products of F^5U. (A) Comparison of the reverse-phase HPLC traces of the TruB (—; 2.42, 2.49 min) and RluA (− −; 3.43 min) products. (B) Comparison of the TruB (− −) and TruA (---) products (retention time = 2.8 min); because of the slight difference in the retention times of U and F^5U (and G and A, not pictured) and the somewhat decreased resolution of the column compared to earlier runs (panels A and C), a coinjection (—) was performed to verify that the TruB and TruA products behaved identically. (C) Comparison of the TruB products (− −; 1.82, 1.92 min) and the RluA products (—; 1.82, 1.95 min) from $[F^5U]$ASL in which C replaces the U that follows F^5U; peaks from the different storage buffers of the two enzymes are marked with asterisks.

Although the technical difficulties of purifying suitable quantities of the adduct between TruA and (full length) $[F^5U]$tRNA precluded the acquisition of NMR spectra of the "TruA products", the lower material demands of mass spectrometry and HPLC analysis (see below) made feasible the analysis of the TruA products by these two methods. The adduct between TruA and $[F^5U]$tRNA was therefore prepared, gel-purified, and heat-disrupted. The RNA was ethanol-precipitated and digested with S1 nuclease and alkaline phosphatase. The nESI-MS analysis of the TruA products reveals them to be the dinucleotides between hydrated F^5U isomers and the C that follows the reactive F^5U in the sequence of $[F^5U]$tRNA.

HPLC Analysis of the F^5U Products. As previously reported, the TruB products are partially resolved under the

HPLC conditions (*9*), but the RluA products elute as a single peak (*12*) with a different retention time than either TruB product (Figure 7, panel A). The TruA products coelute with the TruB products (Figure 7, panel B) as expected from the sequence of $[F^5U]$tRNA, in which C follows F^5U39. Furthermore, the TruB (and therefore, by extension, the TruA products) products coelute with one isomer of photohydrated F^5U. In concordance with the formation of dinucleotide products, the integration of the HPLC traces of component nucleosides in the S1 nuclease/ alkaline phosphatase digestion of $[F^5U]RNA$ after incubation with all three Ψ synthases indicates that the nucleoside following the reactive F^5U falls 1 equiv short of the amount predicted by the sequence (Table 1). Dinucleotide products also explain the previously unsettling ability to detect them by monitoring A_{260}; as expected, the spectra of the product peaks (observed with a diode array detector) match the spectra of the following cytidine or uridine residue (data not shown).

If the differential HPLC retention behavior of the TruB and TruA products relative to the RluA products truly arises from the difference in the 3′ nucleoside of the dinucleotide (C for TruB and TruA; U for RluA), then the mutation of the nucleoside following F^5U to an identical nucleoside should lead to the formation of identical products by the three Ψ synthases. To recognize RNA as a substrate, TruB requires a C to follow the isomerized U (or, in this case, F^5U), which precludes the experiment in that direction (*16*). RluA, however, will accept an RNA with the isomerized U followed by either U or C, so $[F^5U]$ASL with C (in place of U) following the F^5U was incubated with RluA to form an adduct, which was heat-disrupted and digested with S1 nuclease and alkaline phosphatase. As expected, the HPLC analysis revealed products that coelute with TruB products with only a modest difference in the ratio of the major to minor product (Figure 7, panel C).

DISCUSSION

The initial detailed mechanistic investigation of the Ψ synthases was the examination by Santi and co-workers of the
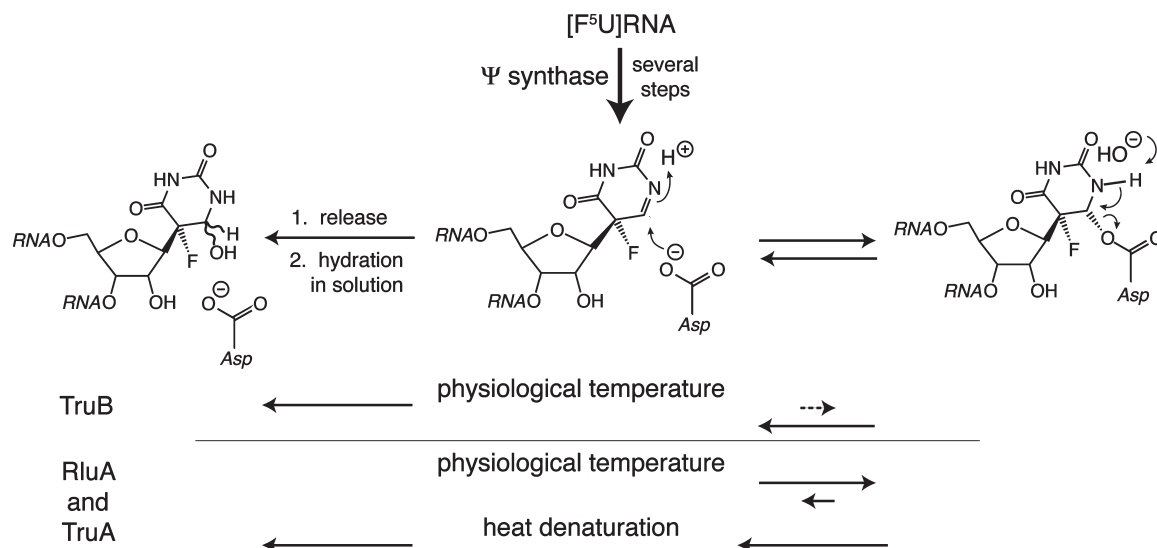
FIGURE 8: A scheme for the handling of F^{5}U in RNA by Ψ synthases. The lower sets of arrows indicate the equilibrium position for adduction and the result of heat disruption for TruB and for RluA and TruA.

interaction between [F^{5}U]tRNA and TruA (formerly Ψ synthase I) (*2*, *7*). The two appeared to form a covalent adduct based on comigration in denaturing gels and irreversible inhibition of enzymatic activity by the fluorinated tRNA. The adduct was heat labile, and the investigators did the maximum characterization of the product of F^{5}U with the limited amount of material available. They demonstrated conclusively that F^{5}U was hydrated during the formation or heat disruption of the adduct. They further used radiolabeled F^{5}U to locate the elution position of the reaction product, which was in a collected fraction that also contained one isomer of photohydrated F^{5}U. On the basis of this evidence, Santi and co-workers very reasonably concluded that TruA follows a mechanism involving a Michael adduct (Figure 1, panel A), with the initial adduct being sufficiently stabilized by the electron-withdrawing properties of the fluoro group so that the enzyme accumulated in this state. Upon heating, hydrolysis of the ester linkage between the active site Asp-60 and F^{5}U was hypothesized to give rise to the hydrated product as depicted in Figure 2 (*7*).

Subsequent studies of the interaction between TruB and RluA and [F^{5}U]RNA revealed that the former does not appear to form a covalent adduct but the latter does (*9*). F^{5}U becomes hydrated by the action of both enzymes, but ^{18}O labeling studies definitively rule out hydrolysis of an ester linkage between the active site Asp and F^{5}U as the mechanism of hydration (*10*, *12*). Furthermore, TruB action generated two products from F^{5}U based on two partially resolved peaks in the reverse-phase HPLC analysis (*9*), but a single peak in the analysis of the RluA products (*12*) suggested that this enzyme generates only one product that differs from either of the TruB products (Figure 7, panel A). Cocrystals of both TruB (*8*) and RluA (*11*) with [F^{5}U]RNA did not show a covalent linkage between RNA and protein, though both could reflect postreaction events rather than productive states.[5] The TruB cocrystal unambiguously showed that the F^{5}U was both rearranged to the *C*-glycoside and hydrated (*8*). Given the high degree of similarity of the active sites of all of the Ψ synthases, it seems unlikely that F^{5}U would be handled so differently to give rise to at least three different

products. The current work extends the ^{18}O labeling studies to TruA to determine whether hydration of F^{5}U occurs by hydrolysis of a Michael intermediate, and the additional characterization of the products of F^{5}U generated by the action of TruA, TruB, and RluA untangles the knot of seemingly conflicting threads of evidence concerning their generation.

^{18}O Labeling. If hydration of F^{5}U occurred because of the hydrolysis of an ester intermediate involving the essential Asp-60 (whether the initial or the rearranged Michael adduct), then oxygen from solvent will be incorporated into Asp-60, and an oxygen atom from the side chain carboxylate group will end in the pyrimidine ring. The results (Figure 4) unambiguously indicate that the pyrimidine ring bears an oxygen atom from solvent and so is directly hydrated. Ester hydrolysis does not occur, a result in agreement with similar studies of TruB and RluA (*10*, *12*).

Unlike in the cases of TruB (which handles [F^{5}U]TSL as a substrate rather than making a tight or covalent adduct) and RluA (which makes a stoichiometric complex that was suitable for direct analysis when 1 equiv of protein is incubated with [F^{5}U]ASL), it was necessary to purify the [F^{5}U]tRNA-TruA adduct from free protein and RNA before analysis. This inconvenience, however, made it trivial to perform an additional experiment: testing whether incorporation of oxygen from solvent occurred during adduct *formation* or during its *disruption*. The incorporation of ^{18}O from solvent into F^{5}U or its rearranged isomer during adduct formation would strongly imply that the adduct does not sport a covalent linkage between RNA and protein but instead is a very tight noncovalent complex. The results show conclusively that the oxygen content of the products of F^{5}U reflects the isotopic composition of the solvent during heat disruption rather than adduct formation.

These results are consistent with the scheme proposed following the TruB and RluA studies in which F^{5}U is rearranged by either mechanism, both of which then falter because the fluoro group cannot be abstracted by an enzymic base in the way that the proton of the natural substrate is (Figure 8) (*10*, *12*). The enzyme binds the rearranged F^{5}U tightly because the sp^3 center at C5 marks it as an intermediate rather than the product (Ψ with a planar pyrimidine ring). The idiosyncratic geometry of some Ψ synthase active sites, such as TruA and RluA, allows the essential Asp to approach C6 closely enough to make a covalent bond,

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[5] The RluA−[F^{5}U]ASL adduct detected in denaturing gels was shown to be sensitive to X-irradiation of its crystal, so the final observed noncovalent complex may be a result of radiolysis.

which is detected by comigration of RNA and protein in denaturing gels; in other Ψ synthases, such as TruB, C6 lies just beyond the reach of the Asp, and no covalent adduct is made. The rearranged intermediate eventually dissociates from TruB and undergoes spontaneous hydration in solution; the isomer with a *cis* relationship between the fluoro group and the new hydroxyl group predominates, as suggested by the appearance of that product in the cocrystal of TruB and [F⁵U]TSL (*8*). Upon heating, the covalent adducts with TruA and RluA suffer elimination (rather than hydrolysis) to regenerate rearranged F⁵U, which undergoes spontaneous hydration once it finds itself in solution as the enzyme denatures. The data currently cannot exclude alternatives, including other idiosyncratic features leading to very tight binding of the rearranged F⁵U by TruA and RluA but not TruB, access of water to the TruB but not TruA or RluA active sites to effect hydration of the bound intermediate, or the greater susceptibility to denaturation by SDS and urea of the complex between TruB and product [F⁵U]RNA. Hydration of the pyrimidine ring of rearranged F⁵U from either face (as shown in Figure 8) is an attractive proposition for a molecule free in aqueous solution and neatly explains the appearance of two isomeric products, but other processes that generate skeletal isomers or other stereoisomers cannot be excluded.

Identity of the Products of F⁵U. The foregoing argument implicitly assumes that the three enzymes convert F⁵U into the same products or at least that RluA and TruA convert F⁵U into one of the two TruB products. The HPLC analyses of the products after enzymatic digestion that nominally would produce nucleoside products suggested that RluA (*12*) formed a single product distinct from the TruB products (*9*), and the TruA product (*7*) was earlier reported as a particular isomer of hydrated F⁵U (with the C−N glycosidic bond intact). These apparent discrepancies were examined in an attempt to resolve them.

Upon large-scale isolation of the TruB products, NMR analysis revealed that the two products of F⁵U were both dinucleotides with the cytidine residue that follows F⁵U in [F⁵U]TSL (Figure 5). This finding was confirmed by mass spectrometry (Figure 6), and a literature search revealed that S1 nuclease has very low activity toward phosphodiester linkages to the 3′ side of nonplanar nucleobases such as a hydrated pyrimidine ring (*17*, *18*). Dinucleotide products of F⁵U immediately explain the disparate HPLC behavior of the TruB and RluA products, for uridine rather than cytidine follows F⁵U in [F⁵U]ASL. Indeed, NMR (Figure 5) and MS (Figure 6) analysis subsequently verified that RluA generates two products that are both dinucleotides with uridine, indicating that the two RluA products comigrate under the HPLC conditions. The ¹H NMR spectra of the TruB and RluA products are very similar (except for the pyrimidine protons of cytidine and uridine), which suggests that both enzymes generate the same two isomeric products from F⁵U itself (only the 3′ nucleoside differs). In agreement with this conclusion, when RluA acts on [F⁵U]ASL in which the uridine following F⁵U was replaced with cytidine, products are obtained that behave identically to the TruB products under the HPLC conditions.

With the resolution of the discrepancy in behavior between the TruB and RluA products, we turned to the TruA products. If all Ψ synthases generate the same products of F⁵U as we hypothesize, then the TruA products should match the TruB products (or, perhaps, only one of them) because the reactive F⁵U in [F⁵U]tRNA is followed by cytidine. The TruA products were prepared on a scale that allowed their detection by absorbance to provide better resolution in the HPLC analysis than was possible with the collection of fractions for scintillation counting used in earlier work. The HPLC (Figure 7) and MS (Figure 6) analysis revealed that TruA does, indeed, generate two products from F⁵U and that they behave identically to the TruB products. One isomer of photohydrated F⁵U migrates very closely to the TruA products (data not shown), which accounts for the earlier report leading to the assignment of the product as a particular isomer of hydrated F⁵U.[6]

Although the definitive characterization of the TruB and RluA products remains a work in progress, several features of the NMR spectra support the conclusion that F⁵U is rearranged in both products. The coupling constants for the 3′ cytidine and uridine components of the dinucleotides match those of the authentic nucleosides free in solution, strongly suggesting that the difference in the two products lies in the F⁵U moiety of each dinucleotide. If one product were hydrated F⁵U rather than its rearranged *C*-glycoside isomer, then a proton would be expected on C5 with *geminal* coupling to the fluoro group, which is on the order of 45−50 Hz (*19*). No proton with such coupling is observed. If one product were rearranged but not hydrated F⁵U, then the proton on C6 would have a chemical shift that reflects its attachment to an sp² carbon, but no such proton is evident. Instead, the resonance of H⁶ in all products has been assigned between 5.20 and 5.34 ppm by a suite of 2D NMR experiments (E. Miracco, unpublished observations). Furthermore, the mass spectra of the TruA, TruB, and RluA products (Figure 6) do not show peaks consistent with rearranged but unhydrated F⁵U, and they all manifest identical and intriguingly unusual fragmentation patterns (B. Bogdanov, manuscript in preparation), further suggesting that all three enzymes handle F⁵U to generate the same products.

CONCLUSIONS

All current data point to the identical handling of F⁵U by TruB, RluA, and TruA to give two isomeric products. Differences in the behavior of those products under identical HPLC conditions is now surely ascribed to the generation of dinucleotide rather than the expected mononucleoside products during enzymatic digestion. The striking similarity of the ¹H NMR spectra of the TruB and RluA products strongly suggests that both of these Ψ synthases generate identical products from F⁵U itself, and the identical behavior of the TruA and TruB products in HPLC and MS analysis suggests that TruA action generates the same products from F⁵U. All of the data are consistent with a mechanism involving a Michael adduct (Figure 1, panel A) or an acylal intermediate (Figure 1, panel B) since both generate rearranged F⁵U. This species can be released into solution (as TruB appears to do) where it undergoes spontaneous hydration, or rearranged F⁵U can (re)combine with the active site Asp to make a covalent adduct (as RluA and TruA appear to do). Upon heating, this adduct suffers elimination to release rearranged F⁵U, which is hydrated spontaneously in solution. Although this scheme (Figure 8) is consistent with the current data, variations

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[6]As explained more fully in the Supporting Information, the NMR spectrum of the isomer of hydrated F⁵U that comigrates with the TruA and TruB products matches that of (5*R*,6*S*)-5,6-dihydro-6-hydroxy-5-fluorouridine (*19*) rather than the 5*S*,6*R* isomer reported by Gu et al. (*7*). Regardless of the assignment of isomers, the comigration of one hydrated F⁵U isomer and the TruA products resolves the apparent discrepancy between the results.

involving hydration at the active site and other stereoisomers (or even skeletal ones) cannot yet be excluded and are the subject of active investigation.

ACKNOWLEDGMENT

SUPPORTING INFORMATION AVAILABLE

Detailed synthetic protocols and characterization for F^5UTP and the photohydration products of F^5U. This material is available free of charge via the Internet at http://pubs.acs.org.

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