Specific Replacement of Functional Groups of Uridine-33 in Yeast Phenylalanine Transfer Ribonucleic Acid†

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ABSTRACT: Functional groups of the highly conserved uridine at position 33 in the anticodon loop of yeast $tRNA^{Phe}$ were altered by a synthetic protocol that replaces U-33 with any desired nucleotide and leaves all other nucleotides of the tRNA intact. The U-33-substituted tRNAs were prepared in an eight-step protocol that begins with partial cleavage of $tRNA^{Phe}$ at U-33 by ribonuclease A. By use of the combined half-molecules as substrate, U-33 was removed from the 5′ half-molecule in three steps and then replaced by using RNA ligase to add the desired nucleoside 3′,5′-bisphosphate. Each position 33 substituted 5′ half-molecule was isolated and annealed to the original 3′ half-molecule from the ribonucelase A digestion. The two halves were then rejoined in three steps to give a full-size $tRNA^{Phe}$ variant. This protocol should be applicable to other RNA molecules where a nucleotide substitution is desired at the 5′ side of an available unique cleavage site. Seven substituted $tRNA^{Phe}$s containing uridine, pseudouridine, 3-methyluridine, 2′-*O*-methyluridine, cytidine, deoxycytidine, and purine riboside at position 33 were assayed for aminoacylation with yeast phenylalanyl-tRNA synthetase. Each of the seven tRNAs aminoacylated normally. Thus, unlike the adjacent guanine residue at position 34, U-33 is not involved in the interaction between yeast $tRNA^{Phe}$ and yeast phenylalanyl-tRNA synthetase.

A uridine at position 33 is a constant feature in the sequence of tRNAs. The occurrence of another nucleotide at this position is extremely rare. In a recent compilation of 260 tRNA sequences from prokaryotic, eukaryotic, mitochondrial, and archaebacterial sources, 253 of them contain uridine at position 33 (Grosjean et al., 1982). All the exceptions have cytidine at position 33, and most of the exceptions are eukaryotic initiator tRNAs. The highly conserved nature of this nucleotide suggests that it may play a crucial role in one of the functions of tRNA.

In each available crystal structure of four tRNAs [Jack et al., 1976; Moras et al., 1980; Quigley & Rich, 1976; Schevitz et al., 1979; Woo et al., 1980; for a review see Wright (1982)] the sugar phosphate backbone turns an abrupt corner just after position 33. The only exception to this conformation is in the crystal structure of a fifth tRNA (yeast $tRNA^{Gly}$), but this tRNA is not in a native conformation (Wright et al., 1979). In the structure of yeast $tRNA^{Phe}$,[1] two hydrogen bonds of U-33 apear to stabilize the sharp turn of the anticodon loop (Quigley & Rich, 1976). One of the bonds extends from the N-3 hydrogen of uracil-33 to an oxygen of phosphate-36, and the other occurs between the 2′-O hydrogen of ribose-33 and N-7 of adenine-35 (Figure 1). Since ψ-55 has a similar conformation in the $tRNA^{Phe}$ TψCG loop, this "U-turn" structure appears to be a common means to alter the direction of an RNA chain. The conformation of U-33 in *Escherichia coli* initiator $tRNA_f^{Met}$ contrasts sharply from yeast $tRNA^{Phe}$ and other tRNAs (Woo et al., 1980). In this structure, the uracil ring is in almost the opposite orientation so that the functional groups of uracil project into the solvent. A hydrogen bond may form between the O-2′ hydrogen of ribose-33 and phosphate-36. Thus, although the nucleotide is conserved, the conformation in that position is not. One structure may be preferred by elongator tRNAs, and the other structure may be preferred for initiator tRNAs (Woo et al., 1980).

The existence of two substantially different conformations for U-33 has led to speculation that an equilibrium between both conformations might exist for all tRNAs (Woo et al., 1980). This hypothesis is indirectly supported by measurements on the fluorescence of Wye base or of ethidium in place of Wye in yeast $tRNA^{Phe}$ (Rigler & Ehrenberg, 1976; Ehrenberg et al., 1979), although the nature of the conformations was not determined. It is not known whether any function of tRNA can be correlated with alternate conformational states of the anticodon loop. Several models for the mechanism of protein synthesis have hypothesized changes in anticodon loop conformation (Woese, 1970; Lake, 1977; Kim, 1978). Fluorescence studies indicate that the aminoacylation reaction also could involve a conformational change of the anticodon loop of a tRNA (Krauss et al., 1976; Ehrlich et al., 1980; Rigler et al., 1981).

In order to examine whether a uridine at position-33 may be necessary for the function of tRNA, we developed a procedure to replace this nucleoside in yeast $tRNA^{Phe}$ with a wide variety of nucleoside analogues. On the basis of the interactions involving U-33 deduced from the crystal structure of yeast $tRNA^{Phe}$, we selected seven nucleosides to replace U-33 (Figure 1). Uridine (U) would serve as a control. Pseudouridine (ψ) would not be expected to disrupt either of the cross-loop hydrogen bonds that involve the N-3 hydrogen of uracil-33 or 2′-O hydrogen of ribose-33 but would slightly alter the uracil moiety. 3-Methyluridine (m^3U) and cytidine (C) would disrupt the hydrogen bond involving the N-3 hydrogen

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[1] Abbreviations: RNA, ribonucleic acid; $tRNA^{Phe}$, yeast phenylalanine transfer RNA; $tRNA^{Phe}$[N-33], $tRNA^{Phe}$ substituted at position 33 with nucleoside N; pNp, nucleoside 3′,5′-bisphosphate; pdC3′p, 2′-deoxycytidine 3′,5′-bisphosphate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, 2-amino-(2-hydroxymethyl)-1,3-propanediol; EDTA, ethylenediaminetetraacetic acid.

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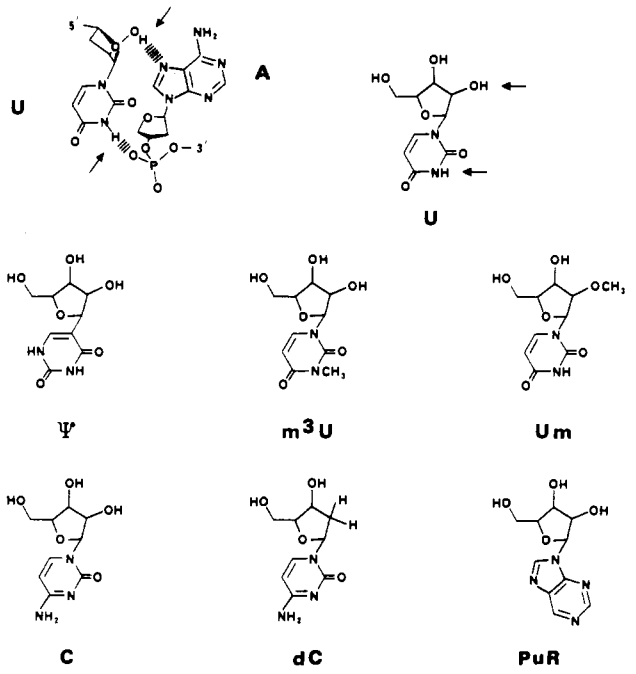

FIGURE 1: Structures of seven nucleosides that were chosen for insertion into position 33 of yeast $tRNA^{Phe}$. An idealized representation of U-33 interactions as proposed by Quigley & Rich (1976) (upper left) is presented for reference. The nucleosides are drawn to emphasize changes of the N3-H and O2′-H functional groups of uridine (arrows).

of uracil-33, 2′-*O*-methyluridine (Um) would disrupt the hydrogen bond involving the 2′-O hydrogen of ribose-33, and 2′-deoxycytidine (dC) would disrupt both hydrogen bonds. Finally, purine riboside (PuR) would be too bulky to fit into the U-turn structure.

Biochemical assays performed on these variant tRNAs should reveal functional consequences of these subtle structural changes. In this paper we report a procedure for substitution of U-33 with the sugar- or base-modified nucleosides and present the results of aminoacylation assays with yeast phenylalanyl-tRNA synthetase.

Materials and Methods

Enzymes. RNA ligase (2200 units/mg) (Moseman-McCoy et al., 1979) and polynucleotide kinase (60 000 units/mg) (Soltis & Uhlenbeck, 1982) were purified from T4-infected *E. coli*. Yeast tRNA nucleotidyltransferase was a gift from P. Sigler. Homogeneous yeast phenylalanyl-tRNA synthetase (500 units/mg) was a gift from P. Remy. Bacterial alkaline phosphatase (BAPF) was purchased from Worthington. Ribonucleases A (90 Kunitz units/mg) and T_1 (5000 units/mg) were purchased from Calbiochem-Behring. Ribonucleases T_2 (1070 units/mg) and P1 (160 units/mg) were purchased from Sigma.

RNAs. Uridine (U), cytidine (C), 3-methyluridine (m^3U), 2′-*O*-methyluridine (Um), and purine riboside (PuR) were purchased from Sigma. Pseudouridine C (β isomer) (ψ) was purchased from Calbiochem-Behring. Nucleoside 3′(2′),5′-bisphosphates were synthesized from the nucleosides by using pyrophosphoryl chloride (Barrio et al., 1978). pdC3′p was purchased from P-L Biochemicals. [γ-^{32}P]ATP was prepared from [^{32}P]orthophosphate (Johnson & Walseth, 1979). [5′-^{32}P]pCp was prepared from [γ-^{32}P]ATP (England et al., 1980). Unfractionated yeast tRNA was a gift from N. Leonard. Yeast $tRNA^{Phe}$ from Boehringer Mannheim (lot 1360137, 1380 pmol/A_{260} unit) was purchased for use as a standard.

Preparation of 5′ Half-Molecule 1–33 and 3′ Half-Molecule 34–74 of $tRNA^{Phe}$. Yeast $tRNA^{Phe}$ was partially purified from unfractionated yeast tRNA with benzoylated DEAE-cellulose (Wimmer et al., 1968). The unfractionated tRNA (5.6 g, 90 000 A_{260} units) was dissolved in 200 mL of 0.4 M NaCl, 10 mM $MgCl_2$, and 50 mM NaOAc, pH 4.5, and applied to a 1-L benzoylated DEAE-cellulose column equilibrated in the same buffer. After the column was washed with 1 L of loading buffer, the majority of the tRNAs was eluted with 5 L of the same buffer containing 1.0 M NaCl. $tRNA^{Phe}$ was eluted in the same buffer containing 1 M NaCl and 20% (v/v) ethanol. The $tRNA^{Phe}$ (3060 A_{260} units) recovered after ethanol precipitation was estimated to be 85% pure by gel electrophoresis (lane b in Figure 3).

A partial digestion of $tRNA^{Phe}$ with ribonuclease A, which maximized the yield of the 5′ half-molecule 1–33, was performed in a 43-mL reaction containing 100 μM partially purified $tRNA^{Phe}$, 0.2 M KCl, 10 mM $Mg(OAc)_2$, 0.1 M Hepes, pH 7.6 at 4 °C, and 20 μg/mL ribonuclease A. After 5.5 h at 4 °C, 1 mL of diethyl pyrocarbonate was added. The reaction was vortexed for 2 min and ethanol precipitated. The inactivated ribonuclease was removed by phenol extraction of the tRNA after resuspension in 12 mL of 5 mM Hepes, pH 7.4, and 10 mM $MgCl_2$. The recovery of total RNA was 3.4 μmol (80%) of equivalent tRNA (lane c in Figure 3).

Since the above digestion conditions give extensive degradation of the 3′ half-molecule 34–74, this fragment was prepared under slightly different reaction conditions. The digestion was performed as above except that the 9-mL reaction contained 50 μM partially purified $tRNA^{Phe}$ and 17 μg/mL ribonuclease A. The incubation was only for 15 min at 4 °C. After treatment with diethyl pyrocarbonate, ethanol precipitation, phenol extraction, and reprecipitation, the reaction mixture was dissolved in 2.0 mL of gel loading buffer (4 M urea, 0.1% xylene cyanol FF, 0.1% bromophenol blue) for purification on two denaturing polyacrylamide gels. A slab gel (330 × 400 × 0.7 mm) contained 20% (w/v) acrylamide, 0.67% (w/v) bis[acrylamide], 7 M urea, 50 mM Tris–borate, pH 8.3, and 1 mM EDTA. A 1-mL sample of the reaction (225 nmol) was applied to a 250 mm wide well in each gel. Electrophoresis was carried out at 1000 V until the xylene cyanol marker reached the bottom of a gel (about 18 h). The band of 3′ half-molecule on each gel was located by UV shadowing, cut out, crushed, and soaked for 1–2 days at 4 °C in 10 mL of soak buffer (0.5 M NH_4OAc, pH 6.5, 10 mM $Mg(OAc)_2$, 0.1 mM EDTA) with constant agitation. After filtration to remove gel particles, the solution was brought to pH 5 with acetic acid and ethanol precipitated. A total of 34 nmol (7.5%) of $tRNA^{Phe}$ 34–74 was recovered.

Substitution of U-33 on the 5′ Half-Molecule. The removal of U-33 from the 3′ terminus of the 5′ half-molecule and the addition of a nucleoside 3′,5′-bisphosphate were carried out on the combined half-molecules in four steps. Dephosphorylation of the terminal phosphates was carried out in a 27-mL reaction containing 107 μM intermediate **2**, 0.4 M NaCl, 10 mM $MgCl_2$, 0.2 M Tris-HCl, pH 8.2, and 64 μg/mL alkaline phosphatase (35 units/mg). After incubation for 23 h at 37 °C, the tRNA was ethanol precipitated. Since the reaction was found to be incomplete when analyzed by gel electrophoresis (see Results), the tRNA was further treated in a 80-mL reaction containing 36 μM **2**, 20 mM $MgCl_2$, 50 mM imidazole hydrochloride, pH 5.9, 3.3 mM dithiothreitol, 10 μg/mL bovine serum albumin, 100 μM ATP, and 150 units/mL polynucleotide kinase. The inherent 3′-phosphatase activity of polynucleotide kinase removes the phosphate while

the ATP acts to stabilize the enzyme (Cameron & Uhlenbeck, 1977). After incubation for 5 h at 37 °C, the reaction was phenol extracted, and intermediate **3** was recovered by ethanol precipitation.

Periodate oxidation was carried out in a 30-mL reaction containing 50 μM **3**, 0.4 M cyclohexylamine hydrochloride 0.1 M Hepes, pH 7.5 at 45 °C, and 7 mM freshly prepared $NaIO_4$. After incubation in the dark for 3.25 h at 35 °C, the reaction was diluted 5-fold with water to increase the solubility of the salts in ethanol and ethanol precipitated. The β-elimination reaction was initiated when the tRNA was resuspended in 38 mL of the same buffer without the $NaIO_4$ and incubated for 72 h at 45 °C. Intermediate **4** was ethanol precipitated.

The terminal phosphate was removed from Cm-32 in a 88-mL reaction containing 10 μM **4**, 0.4 M NaCl, 10 mM $MgCl_2$, 0.2 M Tris-HCl, pH 8.2, and 50 μg/mL alkaline phosphatase (43 units/mg). After 19 h at 37 °C, the turbid solution was phenol extracted, and intermediate **5** was ethanol precipitated.

The addition of each nucleoside 3′,5′-bisphosphate to **5** was carried out in 12-mL reactions containing 3 μM **5**, 200 μM nucleoside 3′(2′),5′-bisphosphate (approximately 100 μM nucleoside 3′,5′-bisphosphate), 200 μM ATP, 50 mM Hepes, pH 8.3 at 16 °C, 20 mM $MgCl_2$, 3.3 mM dithiothreitol, 20% (v/v) dimethyl sulfoxide, 10 μg/mL bovine serum albumin, and 35 μg/mL RNA ligase. After 1 h at 16 °C, each reaction was ethanol precipitated and intermediate **6** resuspended in 1.5 mL of gel loading buffer. The 5′ half-molecule of intermediate **6** for each reaction was purified by gel electrophoresis as was described in the previous section. The modified 3′ half-molecule was discarded. From 3.9 to 4.5 nmol of position 33 modified 5′ half-molecule was recovered from each reaction. This corresponds to a 4.2% yield from the starting material **2**.

Preparation of U-33-Modified tRNA^{Phe}. The modified tRNAs were prepared from half-molecules in three steps. A 1.53-mL reaction contained 2.3 μM modified 5′ half-molecule (residues 1–33), 2.3 μM 3′ half-molecule (residues 34–74), 50 mM Hepes, pH 6.9 at 37 °C, 20 mM $MgCl_2$, 3.3 mM dithiothreitol, and 10 μg/mL bovine serum albumin. After being heated to 70 °C for 5 min, the solution was cooled from 70 to 26 °C over 2 h and from 26 to 0 °C in 40 min. The solution of annealed tRNA half-molecules was made to 6 μM ATP and 150 units/mL polynucleotide kinase and incubated 2 h at 37 °C to form intermediate **7**.

The two half-molecules were joined by adding T4 RNA ligase to the previous reaction to a final concentration of 44 μg/mL, increasing the ATP concentration by 6 μM, and incubating for 4.7 h at 17 °C. The tRNA was ethanol precipitated.

To complete the removal of the 3′-phosphate on C-74 the tRNA was resuspended in a 0.875-mL reaction containing 50 mM imidazole hydrochloride, pH 5.9, 20 mM $MgCl_2$, 3.3 mM dithiothreitol, 10 μg/mL bovine serum albumin, 20 μM ATP, and 300 units/mL polynucleotide kinase. After incubation for 90 min at 36 °C, **8** was ethanol precipitated.

The 3′-terminal CpA was added in a 0.44-mL reaction containing 8 μM **8**, 1 mM ATP, 1 mM CTP, 100 mM Tris-HCl, pH 9.0, 12.5 mM $MgCl_2$, 0.1 mg/mL bovine serum albumin, and enough tRNA nucleotidyltransferase to give complete repair of the 3′ terminus in 2 h at 36 °C. The tRNA was ethanol precipitated and resuspended in 0.375 mL of gel loading buffer. The substituted tRNA was purified on a gel as before except that the gel was 175 × 320 × 0.7 mm, and each substituted tRNA (approximately 3.5 nmol of **9**) was applied to a 60-mm well. Electrophoresis was at 1000 V for 11 h so that the tRNA had migrated about 20 cm. The tRNA was eluted from the gel slice with 6 mL of soak buffer, ethanol precipitated twice with 3 volumes of ethanol and once with 2 volumes of ethanol, and washed with absolute ethanol. Approximately 1.1 nmol of each U-33-modified tRNA was obtained, which corresponds to a 30% yield from the combined half-molecules. For a control, yeast tRNA^{Phe} from Boehringer was repaired on the 3′ terminus and purified by gel electrophoresis in parallel with the substituted tRNAs.

^{32}P Labeling Procedures and Product Identification. The termini of the 5′ and 3′ half-molecules of **2** were dephosphorylated by excising each one from a denaturing gel and incubating with alkaline phosphatase as described above. After phenol extraction and ethanol precipitation, the 5′ terminus of each half-molecule was labeled with [γ-^{32}P]ATP and polynucleotide kinase essentially as described above, and the 3′ terminus of each half-molecule was labeled with [5′-^{32}P]pCp and RNA ligase (England et al., 1980). The 3′ terminus of gel-isolated **9** was ^{32}P labeled in a similar manner. Intermediate **8** with a ^{32}P label in phosphate-34 (arrow in Figure 2) was prepared by including [γ-^{32}P]ATP into the preparation of **7**.

Each ^{32}P-labeled RNA was isolated from a denaturing gel and digested with ribonucleases. Each digest was analyzed by two-dimensional chromatography. Ribonuclease A, T_1, and T_2 digestion was performed at 37 °C for 7 min in 10-μL reactions containing 13 mM NH_4OAc, pH 4.5, 0.12 mg/mL ribonuclease A, 0.12 mg/mL ribonuclease T_1, 1 unit/mL ribonuclease T_2, and enough ^{32}P-labeled RNA to develop an autoradiograph in 2 h. Nuclease P1 digestion was performed at 37 °C for 10 min in 20-μL reactions containing 40 mM NH_4OAc, pH 6, 8 units/mL P1, and ^{32}P-labeled RNA. Two-dimensional chromatography was performed on cellulose thin-layer plates (10 × 10 cm) with isobutyric acid–0.5 M ammonium hydroxide (5:3) in the first dimension, and 2-propanol–HCl–water (70:15:15) in the second dimension (Nishimura, 1979).

Aminoacylation. Reactions (20 μL) to determine the level of aminoacylation contained 160 nM substituted tRNA^{Phe}, 10 μM phenylalanine (20, 37, or 60 Ci/mmol; Amersham), 2 mM ATP, 55 mM KCl, 15 mM $MgCl_2$, 30 mM Hepes, pH 7.0, 4 mM dithiothreitol, and 14 μg/mL phenylalanyl-tRNA synthetase and were incubated 10 min at 37 °C. Reactions (30 μL) to determine aminoacylation rates were performed under the same conditions except that 90 nM tRNA and 0.14 μg/mL enzyme were used. Aliquots (5 μL) were withdrawn at 30-s intervals. Reaction aliquots were quenched in cold 5% TCA (1 mL) and filtered on HAWP nitrocellulose filters (0.45 μm). After five successive washings with cold 5% TCA (1 mL) and a final washing with 95% ethanol (1 mL), the filters were dried, and the tritium was counted in a liquid scintillation counter in 0.4% (w/v) diphenyloxazole in toluene.

Results

Construction of U-33-Substituted Yeast tRNA^{Phe}. The eight-step synthetic scheme for the replacement of U-33 in yeast tRNA^{Phe} is shown in Figure 2. Each step in the synthesis was analyzed by denaturing polyacylamide gel electrophoresis. The stained gels of the intermediates are shown in Figure 3. The starting material for the protocol is tRNA^{Phe}, which has been partially purified from unfractionated yeast tRNA by a simple column procedure. Although the tRNA preparation was not homogeneous when compared to purified yeast tRNA^{Phe} (lanes a and b, Figure 3), the protocol involves

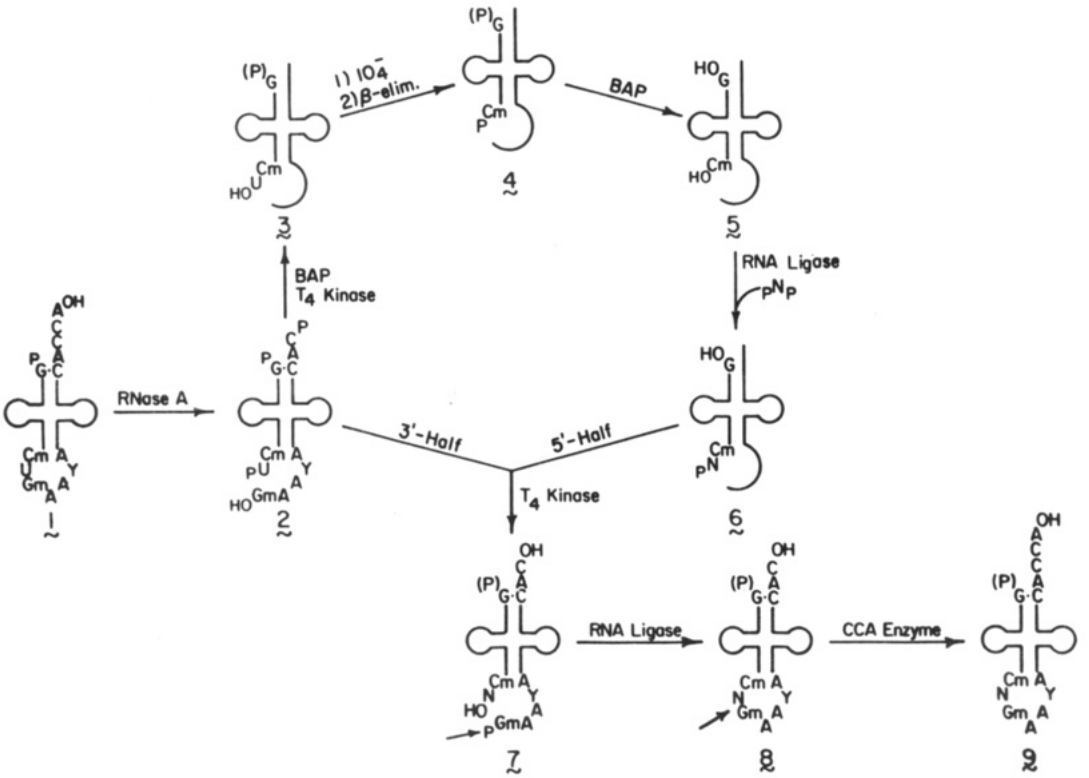

FIGURE 2: Synthetic procedure for construction of a yeast $tRNA^{Phe}$ with an altered U-33. N stands for a replacement nucleoside. Reactions on the 3′ half-molecules of intermediates **3**–**6** are not indicated since this half is subsequently discarded. An internal ^{32}P label can be introduced into intermediate **7** (arrow) with [γ-^{32}P]ATP.

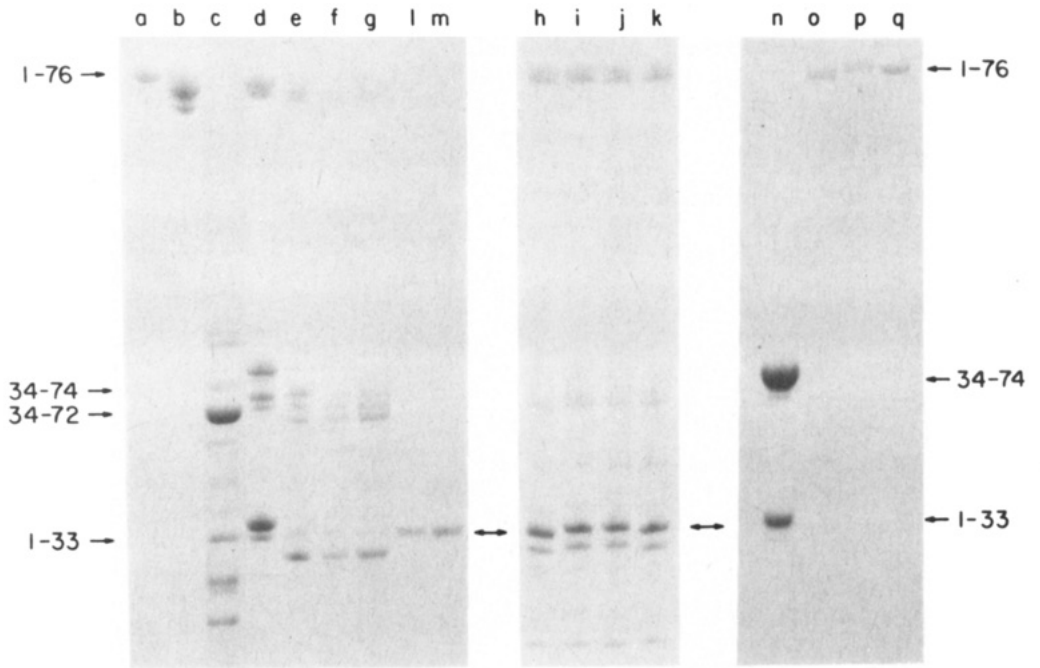

FIGURE 3: Denaturing 20% polyacrylamide gels of synthetic intermediates of U-33-altered variants: (a) $tRNA^{Phe}$ standard, (b) $tRNA^{Phe}$ (**1**) (c) **2**, (d) **3**, (e) **3** after periodate oxidation only, (f) **4**, (g) **5**, (h–k) RNA ligase reactions to form **6** with C, ψ, Um, and PuR at position 33, respectively, (l) gel-purified 5′ half of **2**, (m) gel-purified 5′ half of **6** with U reinserted into position 33, (n) gel-purified 3′ half of **2** and gel-purified 5′ half of **6** with ψ position 33 before addition of T4 kinase, (o) **8** with ψ-33, (p) **9** with ψ-33 before gel purification, and (q) $tRNA^{Phe}$ standard.

additional purification of half-molecules, thereby ensuring a highly purified product.

The partial digestion of $tRNA^{Phe}$ by ribonuclease A was carried out under conditions similar to those of Harbers et al. (1972). Aliquots of the reaction were withdrawn at different times, terminated with diethyl pyrocarbonate, and analyzed by denaturing polyacrylamide gel electrophoresis. Each digestion fragment the size of a tRNA half-molecule was excised from the gel and its 3′ and 5′ nucleotides identified by end labeling and ribonuclease digestion as described under Materials and Methods. These data, combined with the length of the fragment, permitted the identification of the cleavage sites. Ribonuclease A initially cleaves $tRNA^{Phe}$ at U-33 and C-74 at approximately equal rates to produce the desired two tRNA half-molecules 1–33 and 34–74. However, even before the complete disappearance of intact $tRNA^{Phe}$, the 5′ half-

molecule 1–33 begins to be further degraded, and an additional cleavage at C-72 produces 3′ half-molecule 34–72. Thus, careful control of the digestion time is required to optimize the yield of each half-molecule. In lane c of Figure 3, the digestion has been allowed to proceed such that the yield of 5′ half-molecule of intermediate **2** is optimized at the expense of the 3′ half-molecule. A separate reaction, incubated for much shorter times, was required to prepare the 3′ half-molecule 34–74 for use later in the synthesis. For intermediates **3**–**6** the 3′ half-molecle was heterogeneous at the 3′ terminus and, although present in the reactions, was not characterized.

The second step requires the removal of the 3′-terminal phosphate from the 5′ half-molecule of intermediate **2**. When **2** was incubated with bacterial alkaline phosphatase, a substantial proportion of the 3′-phosphate was removed as judged by a small reduction in the mobility of the oligonucleotide (lane d in Figure 3). However even under prolonged incubation, some of the material remained as a faster moving band, which indicated incomplete phosphate removal. Although a subsequent incubation using the 3′-phosphatase activity of polynucleotide kinase increased the yield of dephosphorylated half-molecule, some unreacted half-molecule remained present. The surprising resistance of this terminal phosphate to phosphatase is probably due to the presence of a cyclic 2′,3′-phosphate produced by the partial ribonuclease digestion. Although polynucleotide kinase was recently shown to be able to dephosphorylate terminal cyclic phosphates (T. Weber and O. C. Uhlenbeck, unpublished experiments), the pH of the reaction was not high enough to lead to complete removal.

In the third step U-33 was removed from the 5′ half-molecule by periodate oxidation followed by β-elimination essentially according to the procedure of Sninsky et al. (1976). The oxidation reaction worked well at 37 °C as judged by a substantial increase in mobility of **3** (lane e of Figure 3) as would be expected for the removal of a nucleoside. The subsequent β-elimination step does not result in a mobility shift (lane f of Figure 3) so the end point of the reaction had to be ascertained by the activity of intermediate **4** in subsequent steps. For complete β-elimination, incubation at 45 °C had to be extended to 72 h. The inclusion of 2-aminopyridine (Nishikawa et al., 1982) had no apparent effect on the yield of active intermediate **4**.

The fourth step is removal of the 3′-terminal phosphate from **4** with alkaline phosphatase. Unlike the removal of the 3′-terminal phosphate from **2**, little if any change in the mobility of the oligomer is observed upon dephosphorylation of **4** (lane g, Figure 3). This surprising difference may be a consequence of the borate anion present in the electrophoresis buffer binding better to the 3′-terminal *cis*-diol of **3** than the 3′ terminus of **5**, which has a 2′-*O*-methyl group. The absence of a mobility shift again made it difficult to determine the end point of the reaction except by its activity in the next step.

The fifth step is the joining of a nucleoside 3′,5′-bisphosphate to the 3′-terminal hydroxyl of the 5′ half-molecule of **5**. Analyses of four of these reactions with different nucleotides are shown in lanes h–k of Figure 3. In each case greater than half of **5** is converted to a slower moving band with a mobility identical with that of the original 5′ half-molecule 1–33 (lane l). Under no circumstances was the reaction observed to produce complete conversion to product. The yield of the reaction was independent of the nucleoside 3′,5′-bisphosphate inserted and could not be improved by adding additional RNA ligase. It is likely that the unreacted material contains a blocked 3′ terminus that cannot react with RNA ligase as a result of incomplete reaction in the previous two steps. Elution of the lower band from the gel and reaction with additional RNA ligase and 3′,5′-bisphosphate did not yield additional product. It is interesting to note that the 3′ half-molecule is no longer apparent at this stage of the reaction. The reason for this is unclear but may be due to an exonuclease present in the RNA ligase preparations that caused considerable heterogeneity in the 3′ half-molecule.

The U-33-modified 5′ half-molecule **6** was purified by preparative gel electrophoresis. The resulting material comigrated with the 5′ half-molecule of **2** and was free of the contaminating faster moving band (lane m, Figure 3). Gel-purified 3′ half-molecule 34–74 (lane n, Figure 3) was combined with each modified 5′ half-molecule (as in lanes m and n, Figure 3), heated to 70 °C, and slowly cooled to give a nicked tRNA.

The sixth step involves reacting the combined half-molecules with T4 polynucleotide kinase–3′-phosphatase at pH 6.9, where both activities function. The polynucleotide kinase activity phosphorylates the 5′ end of both half-molecules. In experiments using [γ-^{32}P]ATP in this step, the 3′ half-molecule is phosphorylated more extensively than the 5′ half-molecule. Since the 5′ half-molecule was totally dephosphorylated by alkaline phosphatase in the fourth step as judged by the inability of **5** to add to A_3C with RNA ligase, phosphorylation of the 5′ terminus of the 5′ half-molecule was incomplete as a result of the inaccessability of G-1. The 3′-phosphatase activity of polynucleotide kinase removes the 3′-phosphate from both half-molecules. Although the 5′ half-molecule was rapidly dephosphorylated, the 3′ half-molecule was not as judged by incomplete CCA repair in the last step. It was therefore often necessary to treat with additional 3′-phosphatase after the half-molecules were sealed (see Materials and Methods). Presumably, the resistance of the 3′ half-molecule to dephosphorylation is again a consequence of a terminal cyclic 2′,3′-phosphate that resulted from partial ribonuclease digestion.

The seventh step is joining of the half-molecules of **7** to form a tRNA missing C-75 and A-76. The reaction was performed by adding RNA ligase to the previous reaction mixture. Even though the pH of the reaction was well below the optimum for RNA ligase, very low enzyme concentrations were required for high yields. Since the reaction did not work well unless the half-molecules were annealed, the efficiency of the sealing reaction is a result of the proximity of the two termini. Analysis of the reaction by gel electrophoresis (lane o of Figure 3) showed that both the half-molecules were converted to a slower moving band that migrates close to intact $tRNA^{Phe}$. For most of the U-33-substituted 5′ half-molecules the reaction yield was nearly quantitative. The only exception was when Um was present at position 33 in which case the yield was only about 50%. This result is consistent with an earlier report (Barrio et al., 1978) that oligomers terminating with a 2′-O-methyl are poor RNA ligase acceptors. Surprisingly, the reaction with dC at position 33 went well despite the fact that deoxyribonucleotides are also poor acceptors.

The eighth and final step was to repair the CCA terminus with tRNA nucleotidyltransferase. The final product **9** (lane p, Figure 3) comigrates with native yeast $tRNA^{Phe}$ (lane q, Figure 3) on a denaturing gel. Product **9** was purified by preparative gel electrophoresis, eluted, and precipitated several times with ethanol.

Product Identification. Since the structure of the two half-molecules was established previously, attention was focused on ascertaining that each U-33-substitited $tRNA^{Phe}$ had

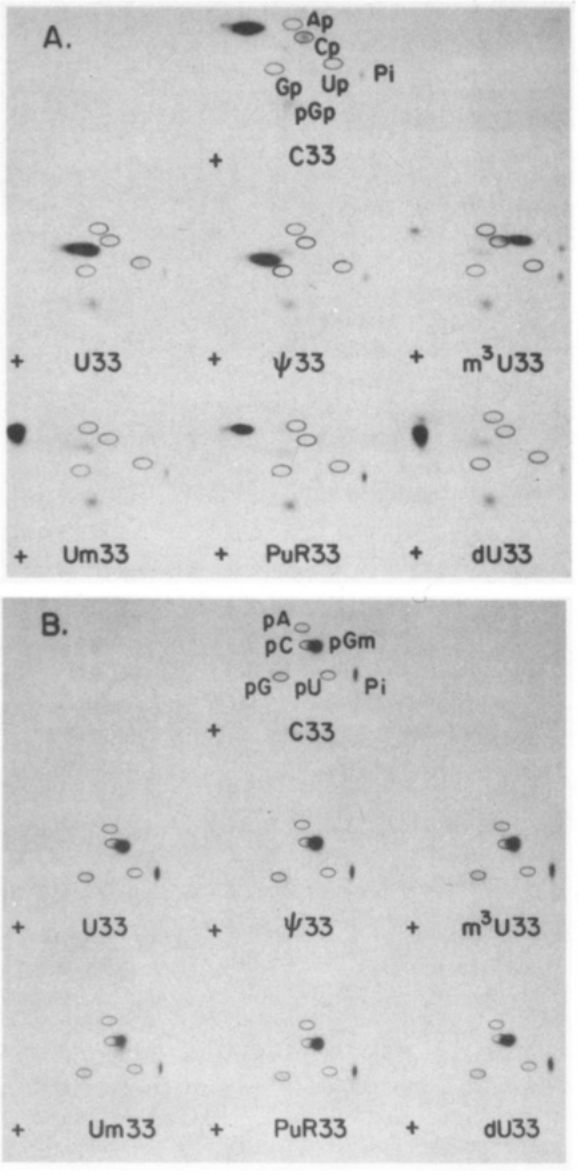

FIGURE 4: (A) Autoradiography of two-dimensional chromatograms of a total RNase A, T_1, and T_2 digest of intermediates **8** with C, U, ψ, m^3U, Um, PuR, and dU at position 33. The intermediates were ^{32}P-labeled at phosphate-34 (arrow Figure 1). The major radioactive spots of **8** with C-33, U-33, and ψ-33 correspond to the expected elution positions of CmCp, CmUp, and Cmψp, respectively. (B) Same as for (A) for a total nuclease P1 digestion of intermediates **8**. The major radioactive spots correspond to the expected elution position of pGm.

the correct internal modification and a properly repaired 3′ terminus. Two experiments suggested that the 3′ terminus was intact. First, a high-resolution RNA sequencing gel showed that the length of each substituted tRNA was identical with that of $tRNA^{Phe}$. Second, each substituted tRNA was 3′ end labeled with [5′-^{32}P]pCp and RNA ligase. After purification and total enzymatic hydrolysis to nucleoside 3′-monophosphates, more than 85% of the radioactivity was Ap as expected for a repaired CCA terminus.

The correct replacement of U-33 in the interior of the molecule by the appropriate modified nucleotide was demonstrated with [γ-^{32}P]ATP in the sixth step to give an intermediate **8** with a ^{32}P introduced into the phosphate between nucleotides 33 and 34. The set of labeled tRNAs in these experiments included $[dU^{33}]tRNA^{Phe}$ and not $[dC^{33}]tRNA^{Phe}$. After purification by gel electrophoresis, each labeled tRNA was digested with either ribonuclease P1 to give nucleoside 5′-monophosphates or a mixture of ribonucleases T_1, T_2, and

Table I: Rates of Aminoacylation of Yeast $tRNA^{Phe}$ Variants

$[N^{33}]tRNA^{Phe}$	fmol/min	relative to $tRNA^{Phe}$
U	50	0.70
Ψ	41	0.57
m^3U	40	0.56
Um	47	0.66
C	61	0.85
dC	63	0.88
PuR	49	0.69
$tRNA^{Phe}$ standard	71 ± 15	1.0 ± 0.2
$tRNA^{Phe}$, gel	43	0.61

A to give di- or tetranucleotides, and the products were separated by two-dimensional thin-layer chromatography. As shown for the products with di- and tetranucleotides in Figure 4A, each anticodon-substituted tRNA gave a major radioactive product and several minor products, which were cut out and counted. The major product, corresponding to greater than 80% of the radioactivity, has a migration position consistent with the correct substitution of the U-33 residue. For example, for $[U^{33}]tRNA^{Phe}$ the product migrates where CmUp is expected, while for $[dU^{33}]tRNA^{Phe}$ the product migrates more slowly in the second dimension as would be expected for CmdUGmAp. Two minor products, P_i and pGp, each appear as about 10% of the total radioactivity in each of the plates in Figure 4A. The P_i is a result of a small amount of phosphatase in the nucleases and the pGp is the result of 5′-phosphorylation of the 5′ half-molecule as discussed previously. If the contributions of these two minor spots are discounted, greater than 90% of the radioactivity in each U-33-substituted tRNA corresponds to the expected product.

As shown in Figure 4B, analysis of the nucleoside 5′-monophosphate derived from each internally labeled U-33-substituted $tRNA^{Phe}$ resulted in the same three radioactive spots. The major spot migrates at the position of pGm, the product expected from the internal ^{32}P label. The minor spots, P_i and pG, appear as a result of phosphatase in the nuclease and a small amount of ^{32}P on the 5′ terminus, respectively, as discussed above. The data in Figure 4 therefore clearly establish that the half-molecules are correctly joined in each U-33-substituted tRNA.

Aminoacylation. Two experiments were carried out to test the activity of $tRNA^{Phe}$ with U, ψ, m^3U, Um, C, dC, and PuR at position 33 for aminoacylation with yeast phenylalanyl-tRNA synthetase. In the first series of reactions a low concentration of each tRNA was incubated with a high concentration of enzyme to determine the maximal level of aminoacylation. Under these conditions, aminoacylation of tRNAs modified at other positions in the anticodon loop would be expected to be complete (Bruce & Uhlenbeck, 1982b). All seven U-33-substituted tRNAs aminoacylated from 55% to 85% of the level of the control $tRNA^{Phe}$. This slight reduction in level of aminoacylation was probably not significant, but rather was a result of contaminating absorbance in the tRNA samples. Thus, all the U-33-substituted tRNAs were active in aminoacylation.

In the second experiment, the kinetics of aminoacylation were measured at a tRNA concentration (90 nM) close to the K_m of yeast $tRNA^{Phe}$ (K_m = 63 nM; Bruce & Uhlenbeck, 1982b) so that a difference in either K_m or V_{max} for a given variant would be clearly detected as a rate change. As shown in Table I, all the U-33-substituted tRNAs aminoacylated at similar rates. None of the rates relative to $tRNA^{Phe}$ standard were more than 10% lower than the rate of gel-eluted $tRNA^{Phe}$ used as a control. Although the precision of the data is low due to limited material available, it is clear that U-33 does

not substantially affect the rate of aminoacylation.

Discussion

The protocol for the synthesis of U-33 variants has three important features. First, the availability of a ribonuclease A cleavage site adjacent to U-33 permits specific access to this nucleotide, allowing its removal and replacement without disturbing the remainder of the tRNA. Thus, all the modified nucleotides in tRNA are unaffected by the procedure. The specificity of the cleavage is greatly aided by the tertiary structure of the tRNA molecule. The only other site of ribonuclease A cleavage removes two nucleotides from the 3′ terminus, which was easily repaired. A second feature of the protocol is that all the steps are carried out on annealed half-molecules. When the tertiary structure of $tRNA^{Phe}$ is maintained, the polynucleotide chain is protected from cleavage during the multiple chemical and enzymatic steps. In addition, undesired intra- and intermolecular joining reactions could occur if purified 5′ half-molecules were used. A third feature of the protocol is that a wide variety of different uridine analogues can be inserted for U-33. This generality is made possible by the broad substrate specificity of RNA ligase for nucleoside 3′,5′-bisphosphates as donors in a joining reaction (Gumport & Uhlenbeck, 1981) and by the facile preparation of these nucleotides from standard and rare nucleosides (Barrio et al., 1978).

It is likely that a similar series of reactions could be carried out on other RNA molecules where a nucleotide substitution is desired at the 5′ side of an available cleavage site. The strength of the procedure is that deletions or substitutions of individual atoms of a particular nucleotide can be made. This permits analysis of the functional consequences of structural changes that are much more detailed than those attainable by mutation or by chemical modification of the entire RNA molecule.

All six U-33-substituted $tRNA^{Phe}$ variants aminoacylated at a rate very similar to unaltered $tRNA^{Phe}$. Even the variants with the most extreme structural changes, $[PuR^{33}]tRNA^{Phe}$ and $[dC^{33}]tRNA^{Phe}$, had rates of aminoacylation within 30% of $[U\text{-}33]tRNA^{Phe}$. This result is surprising since a purine base bears little chemical similarity to a uracil and dC differs substantially from U in hydrogen-bonding functionality. Thus, U-33 does not influence the interaction between yeast $tRNA^{Phe}$ and yeast phenylalanyl-tRNA synthetase. These data contrast sharply with similar data obtained with variants of $tRNA^{Phe}$ at positions 34–37 (Bruce & Uhlenbeck, 1982b). Substitution of Gm-34 by another nucleotide reduced the aminoacylation rate approximately 10-fold while changes at positions 35–37 show 2–5-fold reductions in rate. Thus, the current work has defined a boundary of the anticodon region on $tRNA^{Phe}$ that contacts the synthetase.

The data can be rationalized if the synthetase interacts with an anticodon loop structure very similar to the one found in the $tRNA^{Phe}$ crystal structure. In the crystal structure, the functional groups of the anticodon nucleotides project toward the inside of the "L" formed by the tRNA molecule in such a way that they could contact the enzyme. In contrast, U-33 is on the outside of the L and faces toward the interior of the anticodon loop, which could reduce access to the enzyme. This rationalization is supported by other evidence that suggests that synthetase binding occurs only along the "inside" of the tertiary structure of $tRNA^{Phe}$ [for reviews, see Schimmel (1977, 1980) and Schimmel & Söll (1979)].

It is interesting that the cross-loop hydrogen bonds in the "U-turn" between U-33 and the anticodon do not appear necessary for the proper interaction of tRNA with synthetase. Certainly, $[PuR^{33}]tRNA^{Phe}$ and $[dC^{33}]tRNA^{Phe}$ could not form these hydrogen bonds for the reasons discussed above. This observation indicates that while the synthetase may recognize the general tertiary structure of the anticodon loop in the crystal, the conformation of the anticodon region does not have to be exactly the same. Thus, different conformations of U-33 such as found between the crystal structures of yeast $tRNA^{Phe}$ and *E. coli* $tRNA_f^{Met}$ may not be important for the interaction of yeast $tRNA^{Phe}$ and yeast phenylalanyl-tRNA synthetase.

It is quite possible that U-33 is important in one or more steps of the translaton mechanism. Although U-33 is known not to be essential for the suppression of amber codons in vitro (Bare et al., 1983) or in vivo (Thompson et al., 1982), substitution of the nucleotide leads to a reduced efficiency of suppression. The availability of U-33-substituted $tRNA^{Phe}$ variants should permit evaluation of the importance of this nucleotide in biochemically well-defined poly(U)-directed translation systems. In collaboration with D. Dix and R. Thompson, we have recently shown that $[PuR^{33}]tRNA^{Phe}$ and $[dC^{33}]tRNA^{Phe}$ have decreased activities in a single-turnover kinetic assay.

Registry No. U, 58-96-8; RNase, 9001-99-4; Ψ, 1445-07-4; m^3U, 2140-69-4; C, 65-46-3; Um, 2140-76-3; dC, 951-77-9; PuR, 550-33-4; alkaline phosphatase, 9001-78-9; polynucleotide kinase, 37211-65-7; RNA ligase, 37353-39-2; tRNA nucleotidyltransferase, 9026-11-3.

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Kinetics of Open Complex Formation between *Escherichia coli* RNA Polymerase and the *lac* UV5 Promoter. Evidence for a Sequential Mechanism Involving Three Steps†

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ABSTRACT: The forward and reverse kinetics of open complex formation between *Escherichia coli* RNA polymerase and the *lac* UV5 promoter have been studied in the temperature range of 15-42 °C. The standard two-step model, involving the formation of a closed intermediate, RP_c, followed by an isomerization that leads to the active complex RP_o, could not account for the present data. The promoter-enzyme lifetime measurements showed an inverse temperature dependence (apparent activation energy, −35 kcal/mol). A third step, which is very temperature dependent and which is very rapid at 37 °C, was postulated to involve the unstacking of DNA base pairs that immediately precedes open complex formation. Evidence for incorporating a new binary complex, RP_i, in the pathway was provided by experiments that distinguished between stably bound species and active promoter after temperature-jump perturbations. These experiments allowed measurement of the rate of reequilibration between the stably bound species and determination of the corresponding equilibrium constant. They indicated that the third step became rate limiting below 20 °C; this prediction was checked by an analysis of the forward kinetics. A quantitative evaluation of the parameters involved in this three-step model is provided. Similar experiments were performed on a negatively supercoiled template: in this case the third equilibrium was driven toward formation of the open complex even at low temperature, and the corresponding step was not rate limiting.

It is generally postulated that the formation of an active complex between *Escherichia coli* RNA polymerase and a bacterial promoter involves at least two steps before triphosphate binding and chain initiation. An inactive binary complex is first formed: this transient species, the closed complex, undergoes a slow and thermodynamically favored isomerization to the transcriptionally active complex [cf. Walter et al. (1967) and Chamberlin (1974)]. This two-step model can be summarized as shown in Scheme I.

Scheme I

$$R + P \underset{K_B}{\rightleftharpoons} RP_c \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} RP_o$$

For a given promoter, it is now possible to determine the rate of formation of the open complex RP_o through a simple enzymatic assay. This rate depends on the initial concentration

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