Enzymatic Replacement of the Anticodon of Yeast Phenylalanine Transfer Ribonucleic Acid†

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ABSTRACT: An efficient procedure for the replacement of the anticodon and the adjacent hypermodified nucleotide (residues 34–37) of yeast $tRNA^{Phe}$ with any desired oligoribonucleotide sequence has been developed. The four residues are removed by chemical cleavage at Y-37 and partial ribonuclease A digestion at U-33. An oligonucleotide is inserted in three steps by using T4 RNA ligase and T4 polynucleotide kinase. When different oligonucleotides are inserted, both the size of the loop and the sequence of nucleotides in the anticodon region of this tRNA can be varied. The ability of the different anticodon loop substituted tRNAs to be aminoacylated by yeast phenylalanyl-tRNA synthetase is dependent upon the sequence of the oligonucleotide inserted. This suggests that there is an important interaction between the anticodon region of yeast $tRNA^{Phe}$ and its synthetase.

The use of T4 RNA ligase has greatly increased our ability to manipulate sequences of RNA.[1] Not only can oligoribonucleotides be joined to form larger molecules (England & Uhlenbeck, 1978a; Ohtsuka et al., 1980) but reactions with intact RNA molecules are also possible. RNA ligase has been used to modify the 3′ and 5′ termini of tRNA (Bruce & Uhlenbeck, 1978; Hecht et al., 1978) and the 3′ termini of a variety of larger RNAs (England & Uhlenbeck, 1978b). Alterations of the termini of 5S RNA have allowed the study of the specificity of a RNA processing enzyme (Stahl et al., 1980). Modifications at internal positions are also possible where methods for specific cleavage are available. Kaufmann & Littauer (1974) were able to construct, in low yield, a $tRNA^{Phe}$ molecule with a single nucleotide removed from the anticodon loop. In this paper we describe a procedure for the removal of the anticodon and the hypermodified nucleotide from yeast $tRNA^{Phe}$ and the replacement of these four nucleotides with a new oligoribonucleotide. Yields in excess of 40% can be obtained with this method.

The removal of the tetranucleotide from the anticodon loop produces tRNA fragments which aminoacylate poorly with yeast phenylalanyl-tRNA synthetase (PRS). Replacement of the excised nucleotides can restore the aminoacylation activity to normal; however, some anticodon substituted tRNAs are poor substrates. This indicates that the nucleotides in the anticodon loop are involved in the specific interaction of yeast $tRNA^{Phe}$ with its cognate synthetase.

Materials and Methods

Enzymes. T4 RNA ligase (specific activity 2000 units/mg) was purified from *Escherichia coli* infected with T4 *am* 4314 (Moseman-McCoy et al., 1979). T4 polynucleotide kinase (Cameron & Uhlenbeck, 1977), rabbit liver tRNA nucleotidyltransferase (Deutscher & Masiakowski, 1978), and partially purified yeast phenylalanyl-tRNA synthetase (Leisch, 1977) were gifts from D. Soltis, M. Deutscher, and J. Leisch, respectively. Bacterial alkaline phosphatase (BAPF) was purchased from Worthington, and ribonuclease A was purchased from Sigma. These latter two enzymes were stored as 1 mg/mL solutions in 0.1 M NaCl, 10 mM $MgCl_2$, and 10 mM Tris-HCl (pH 7.4).

RNAs. Yeast $tRNA^{Phe}$ was purchased from Boehringer Mannheim (lot 1199234; 1149 pmol/A_{260} unit). [γ-^{32}P]ATP was prepared from [^{32}P]orthophosphate (New England Nuclear) by the method of Johnson & Walseth (1979), and [5′-^{32}P]pCp was prepared as described by England et al. (1980). $(Ap)_3C$ and [^{32}P]$(Ap)_3C$ were prepared as described previously (Bruce & Uhlenbeck, 1978). $(Ap)_{13}A$ and $(Cp)_nG$ were prepared as described by de Haseth & Uhlenbeck (1980) and England & Uhlenbeck (1978a). $G(pA)_n$ was prepared by an equilibrium reaction with polynucleotide phosphorylase (Thach & Doty, 1965), and GpApApG was prepared by the addition of pGp to $G(pA)_2$ with RNA ligase (England & Uhlenbeck, 1978a).

Gel Electrophoresis. Denaturing polyacrylamide gels (270 × 150 × 0.75 mm) contained 20% (w/v) acrylamide (Aldrich), 0.67% (w/v) bis(acrylamide), 7 M urea, 50 mM Tris–borate, pH 8.3, and 1 mM EDTA (Donis-Keller et al., 1977). Electrophoresis was usually carried out at 1000 V for 3 h. Analytical gels were stained with "Stains All" (Eastman) according to the procedure of Dahlberg et al. (1969) and then autoradiographed by using Kodak Omat-R X-ray film. The same type of gel was used for the purification of anticodon-substituted tRNAs. In this case the (3 × 10 mm) tRNA band was located by UV shadowing, cut out with a razor blade, crushed, and soaked overnight at 4 °C in 0.4 mL of 50 mM NaOAc (pH 4.5), 1 mM EDTA, and 0.1% $NaDodSO_4$. After centrifugation to remove the acrylamide fragments, 3 volumes of ethanol was added to the tRNA, the solution was cooled to −70 °C for at least 2 h, and the tRNA was precipitated by centrifugation in an Eppendorf microfuge for 5 min. The tRNA was resuspended in 0.3 M NaOAc (pH 4.5) and reprecipitated to ensure removal of the $NaDodSO_4$.

Aminoacylation Kinetics. Reactions (50 μL) contained 0.04–0.35 μM tRNA, 20 μM L-[^{3}H]phenylalanine (Amersham, 5 Ci/mmol), 1 mM ATP, 25 mM KCl, 15 mM $MgCl_2$, 25 mM Tris (pH 7.5), 60 mM β-mercaptoethanol, 30 μg/mL BSA, and sufficient yeast PRS to give linear kinetics for 5 min at 37 °C. Time points (10 μL) were spotted on Whatman

† From the Department of Biochemistry, University of Illinois, Urbana, Illinois 61801. *Received August 17, 1981.* Presented in part at the International Union of Biochemists Meeting in Toronto in July 1979, abstract 02-5-H57. This work was supported by a grant from the National Institutes of Health (GM 19059).

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[1] Abbreviations: RNA, ribonucleic acid; $tRNA^{Phe}$, yeast phenylalanine tRNA; PRS, yeast phenylalanyl-tRNA synthetase; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, 2-amino-(2-hydroxymethyl)-1,3-propanediol; EDTA, ethylenediaminetetraacetate; $NaDodSO_4$, sodium dodecyl sulfate; PPO, 2,5-diphenyloxazole.

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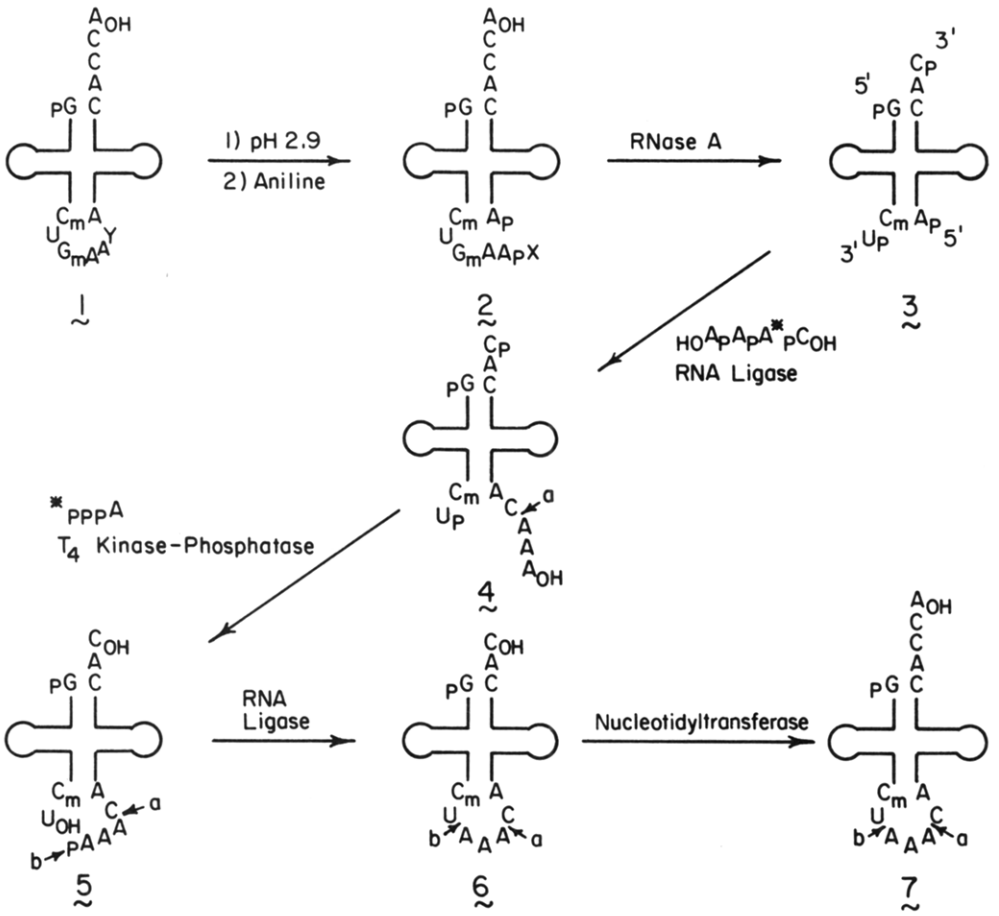

FIGURE 1: Steps involved in the construction of a $tRNA^{Phe}$ modified in the anticodon. An internal ^{32}P label can be introduced at position a with internally labeled $(Ap)_3C$ and at position b with [γ-^{32}P]ATP.

3MM squares and dropped into cold 5% trichloroacetic acid. The squares were washed 5 × 200 mL with 5% Cl_3CCOOH, 1 × 200 mL with ethanol, and 1 × 50 mL with ether. The dried squares were counted in a liquid scintillation counter in PPO–toluene.

Results

Construction of Anticodon-Substituted $tRNA^{Phe}$. The six steps required for the replacement of residues 34–37 of yeast $tRNA^{Phe}$ with the tetranucleotide $(Ap_3)C$ are summarized in Figure 1. Each step in the reaction was analyzed by denaturing polyacrylamide gel electrophoresis. The stained gels and their autoradiographs are shown in Figure 2.

The first step involves the specific depurination of the hypermodified base Y-37 followed by chemical chain scission at this position (Theibe & Zachau, 1971). The $tRNA^{Phe}$ was dissolved in distilled water to 1 mg/mL and brought to pH 2.9 with 0.1 N HCl. After 3 h at 37 °C, the $tRNA^{Phe}_{-Y}$ was purified from any unreacted $tRNA^{Phe}$ on a benzoylated DEAE-cellulose column according to the procedure of Wimmer et al. (1968). Although the yield of this step is generally very high (>95%), column purification at this stage ensures that no intact $tRNA^{Phe}$ will contaminate the final product. After dialysis, concentration, and ethanol precipitation, the $tRNA^{Phe}_{-Y}$ was resuspended to 1 mg/mL in 0.45 M aniline hydrochloride (pH 4.5) and incubated for 5 h at 20 °C (Harbers et al., 1972). This reaction quantitatively cleaves the chain to give the tRNA half-molecules 1–36 and 38–76 (intermediate **2** in Figure 1). The two halves, presumably still paired with one another, were recovered by ethanol precipitation. Depurination and chain scission by these procedures do not lead to the complete removal of ribose-37 from the 5′ half-molecule. This led to very low yields when the resealing of these half-molecules was attempted (Kaufmann & Littauer, 1974).

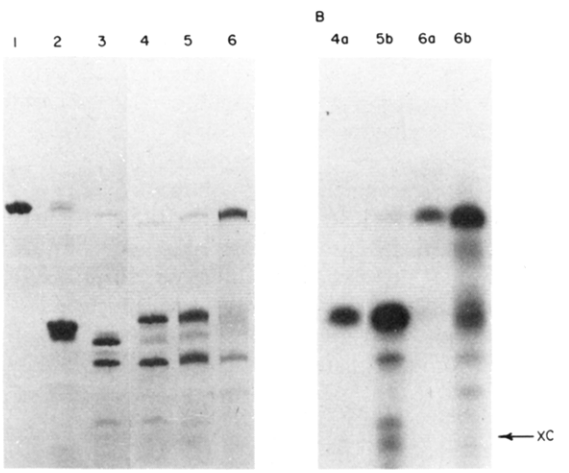

FIGURE 2: (A) Denaturing 20% polyacrylamide gel of intermediates **1–6** stained with "Stains All". (B) Autoradiographs of wells 4–6 of (A), with a and b indicating the position of the label.

The second step is a partial digestion of intermediate **2** with ribonuclease A. This causes cleavage at U-33 resulting in the removal of the anticodon G_mAA (residues 34–36) as well as cleavage at C-74 resulting in the removal of C-75 and A-76. The conditions for this reaction are similar to those of Harbers et al. (1972) except that the anticodon loop of intermediate **2** is considerably more sensitive to ribonuclease A than that of $tRNA^{Phe}$ or $tRNA^{Phe}_{-Y}$. A reaction containing 0.5 mg/mL intermediate **2**, 0.1 M Tris-HCl (pH 7.5), 0.2 M KCl, 10 mM

MgOAc, and 1 μg/mL ribonuclease A was incubated for 1 h at 0 °C. The ribonuclease A was inactivated by adding diethyl pyrocarbonate to 1.5% (v/v). The reaction was vortexed for 1 min, and immediately ethanol precipitated. The products of this digestion (intermediate **3**) are the half-molecules 1–33 and 38–74 with both 3′- and 5′-terminal phosphates. As displayed in well 3 of Figure 2A, the two half-molecules are well resolved, with the upper band being the 3′ half-molecule (37 residues) and the lower band being the 5′ half-molecule (33 residues).

These assignments were confirmed by determining the 3′- and 5′-terminal nucleotides of both bands. This was done by treating intermediate **3** with bacterial alkaline phosphatase and labeling the 5′ ends with [γ-^{32}P]ATP and polynucleotide kinase or the 3′ ends with [5′-^{32}P]pCp and RNA ligase. The labeled half-molecules were purified by gel electrophoresis, cut out of the gel, and soaked for 12 h in 0.3 M KOH. The hydrolyzed 5′-end-labeled bands were analyzed by poly(ethylenimine) thin-layer chromatography with 0.8 M $(NH_4)_2SO_4$, and the hydrolyzed 3′-end-labeled bands were analyzed by high-voltage paper electrophoresis (Greenfield et al., 1975) (data not shown). The upper band had a 5′-terminal A and a 3′-terminal C, as expected for the 3′ half-molecule. The lower band had a 5′-terminal G and a 3′-terminal C_mU as expected for the 5′ half-molecule. The combined half-molecules (intermediate **3**) were generally prepared in large amounts (10 mg) and stored frozen in distilled water or 10 mM Hepes (pH 7.5).

The third step is the addition of the oligonucleotide acceptor $(Ap)_3C$ to the 5′-terminal phosphate of the 3′ half-molecule to produce intermediate **4**. The conditions used for this reaction were 8 μM [^{32}P]$(Ap)_3C$, 3 μM intermediate **3**, 5 μM ATP, 20 mM $MgCl_2$, 3.3 mM dithiothreitol, 50 mM Hepes, pH 7.5, 10 μg/mL bovine serum albumin, and 10 units/mL T4 RNA ligase. After incubation for 4 h at 4 °C, the reaction was terminated by heating 3 min at 65 °C and ethanol precipitation. Analysis of this reaction by gel electrophoresis (well 4, Figure 2A) shows that greater than 90% of the 3′ half-molecules (upper band) is converted to a slower moving band with a mobility consistent with the addition of four nucleotides. Autoradiography (well 4a, Figure 2B) reveals that the ^{32}P-labeled $(Ap)_3C$ is incorporated only into this band as expected. This reaction can therefore be carried out on the combined half-molecules without any detectable addition to the 3′-terminal phosphate of the 5′ half-molecule. This is consistent with our previous observation that the 5′-terminal phosphate of the intact tRNA^{Phe} is not an effective donor in a RNA ligase reaction, presumably due to the fact that G-1 is base paired (Bruce & Uhlenbeck, 1978).

We have studied this addition reaction with a variety of oligonucleotides. Consistent with other oligomer joining reactions (England & Uhlenbeck, 1978a), the oligomer must be at least three residues long for successful addition. Furthermore, the rate and yield of the reaction depend upon the identity of the oligomer added. For example, for comparably high yields with the poor acceptor $(Up)_3U$, 75 units/mL RNA ligase is required at the above conditions. Preliminary studies also indicate that the addition reactions can be carried out at substantially (10-fold) higher concentrations of substrates without a corresponding increase in enzyme concentration.

The fourth step is the removal of the 3′-terminal phosphates from both half-molecules and the phosphorylation of the 5′ terminus of the oligonucleotide added in the previous step. At pH 6.9, polynucleotide kinase with its associated 3′ phosphatase activity (Cameron & Uhlenbeck, 1977) can catalyze both these reactions. The product of the previous step (intermediate **4**) was resuspended at a concentration of 3 μM in buffer containing 30 μM ATP, 20 mM $MgCl_2$, 3.3 mM dithiothreitol, 50 mM Hepes (pH 6.9), and 10 μg/mL bovine serum albumin and incubated with 50 units/mL polynucleotide kinase for 1 h at 37 °C. If [γ-^{32}P]ATP is included in the reaction, the phosphorylation of the 3′ half (upper band) can be detected by autoradiography (well 5b, Figure 2B). Since no change in the number of nucleotides occurred, little change in the positions of the stained bands is observed.

The fifth step, joining the half-molecules to form intermediate **6**, was carried out by simply adding RNA ligase to the previous reaction to a final concentration of 4 units/mL and incubating for an additional hour at 37 °C. The enzymes were then inactivated by heating to 65 °C for 3 min, and the tRNA was ethanol precipitated. Analysis of the reaction by gel electrophoresis (well 6, Figure 2A) shows that both half-molecules have been converted to a slower moving band which migrates close to intact tRNA^{Phe}. Autoradiographs of reactions with ^{32}P introduced in the oligomer or from ATP also show that the labeled 3′ half-molecule has been converted to a molecule of tRNA size (well 6a,b, Figure 2B). Although this joining reaction is, for convenience, carried out at a pH well below the optimum for RNA ligase, very low enzyme concentrations are required for high yields. The precise amount required is primarily dependent upon the length of the oligonucleotide inserted. For example, when $(Ap)_3C$ was inserted to form an anticodon loop of seven residues, 4 units/mL RNA ligase was sufficient. However, when $(Ap)_{13}A$ was used to make a 17-residue anticodon loop, 40 units/mL RNA ligase was needed. On the other hand, if intermediate **3** is treated with polynucleotide kinase and one attempts to seal the half-molecules to form a putative anticodon loop of only three residues, very little product is formed, even with very high levels of RNA ligase. These observations suggest that the joining of the half-molecules by T4 RNA ligase is efficient due to the close proximity of the donor phosphate and acceptor hydroxyl when the halves are base paired. When the anticodon loop to be sealed is either shorter or longer, the termini are in less favorable proximity, and more enzyme is needed. In order to avoid intermolecular reactions between the 3′ terminus of the tRNA and small oligonucleotides in the reaction, it is important not to add a large excess of RNA ligase in this step.

The final step is the repair of the CCA terminus. Incubation of 10 μM intermediate **6**, 0.5 mM ATP, 0.1 mM CTP, 10 mM $MgCl_2$, 1 mM dithiothreitol, 10 mM Tris-HCl (pH 8.2), and 0.3 unit/mL rabbit liver tRNA nucleotidyltransferase for 1 h at 20 °C replaces the two 3′-terminal nucleotides removed by the ribonuclease A digestion (step 2). The final product (**7**) is then purified on a 20% denaturing polyacrylamide gel as described under Materials and Methods.

Although the procedure to replace nucleotides 34–37 in yeast tRNA^{Phe} is relatively complex, the yields at each step are quite high, and the methods are relatively simple since there is no requirement for purification of the intermediates other than ethanol precipitation. Thus it is possible to obtain overall yields (steps 1–7) of greater than 40%. We have successfully used this procedure to insert more than 30 different oligonucleotides into the anticodon loop of tRNA^{Phe}. These include $(Cp)_{2-7}G$, $(Cp)_{11}G$, $(Ap)_{13}A$, $(Ap)_{22}A$, UpUpApA, UpCpApA, CpUpApA, GpApApG, $(Ap)_3G$, $G(pA)_3$, CpUpCpU, GpUpUpApA, $(Ip)_3I$, CpCpUpU, $C(pU)_3$, GpUpCpApA, GpCpUpApA, and GpUpCpG. As noted previously, different T4 RNA ligase concentrations in steps

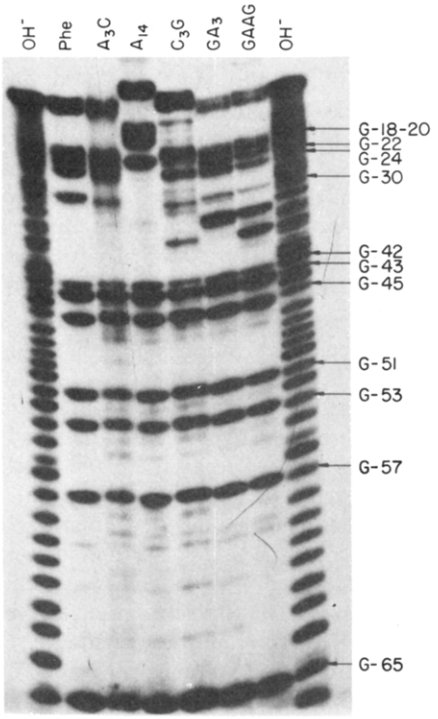

FIGURE 3: Partial T_1 digests of $tRNA^{Phe}$ (marked Phe) and five anticodon-substituted tRNAs (marked by the oligomer substituted) labeled on their 3′ terminus with [5′-^{32}P]pCp separated on a 20% RNA sequencing gel and autoradiographed. A partial base hydrolysis (marked OH^-) indexes the chain lengths, and the numbers indicate the positions of the guanosines in $tRNA^{Phe}$.

3 and 5 were required for different oligomer lengths and compositions. However, the procedure appears to be quite general for different oligomer sequences.

Product Identification. Three different anticodon-substituted $tRNA^{Phe}$ molecules [$(Ap)_3C$, $(Cp)_3G$, and $(Ap)_{13}A$] were 3′ end labeled with [5′-^{32}P]pCp and RNA ligase (Bruce & Uhlenbeck, 1978), and their sequences were determined by the direct chemical method (Peattie, 1979). These data (not shown) confirmed that the oligonucleotides were accurately inserted into the $tRNA^{Phe}$ anticodon loop, and no other changes in the molecule were observed. This point is also well demonstrated in Figure 3 where a partial ribonuclease T_1 digestion (Donis-Keller et al., 1977) of 3′-end-labeled $tRNA^{Phe}$ is compared with the partial T_1 digestion of five different 3′-end-labeled anticodon-substituted tRNAs on a 20% polyacrylamide sequencing gel. The 3′- and 5′-terminal portions of all six tRNAs have identical patterns from the T_1 digestion, although the 5′-terminal portion of the $tRNA_{A_{14}}$ is shifted due to the longer anticodon loop. However, within the large T_1-resistant region between G-30 and G-42 which includes the anticodon loop of $tRNA^{Phe}$, the expected differences occur. When $(Ap)_3C$ is inserted, the length of the region remains the same, and no additional bands appear. When $(Ap)_{13}A$ is inserted, the length of the region increases appropriately, and no additional bands appear. However, when the tetramers $(Cp)_3G$, $G(pA)_3$, or GpApApG are inserted, the length of the region remains the same, but additional T_1 cleavages are observed at the appropriate places where a guanosine occurs within the oligomer. Thus, RNA sequencing data confirm accurate anticodon substitution.

Since partial digestion data do not give an accurate measure of the purity of the gel-eluted product, two additional analyses were done. In the first, several of the 3′-end-labeled anticodon-substituted tRNAs were subjected to total base hydrolysis and analysis of the ^{32}P nucleotides by high-voltage electrophoresis. In each case, more than 90% of the label appeared as [3′-^{32}P]Ap, confirming that the CCA terminus was repaired. In a second experiment, a preparation of intermediate **6** with a ^{32}P label introduced with [γ-^{32}P]ATP (position b) was purified by gel electrophoresis, hydrolyzed by ribonucleases T_1, T_2, and A, and analyzed by two-dimensional chromatography on a poly(ethylenimine) thin-layer plate (Nishimura, 1979). In Figure 4, the autoradiograph of this plate indicates a single product corresponding to the expected position of C_mpUp (Nishimura, 1979). Thus the sealing of the half-molecules at position 33 is accurate and quantitative.

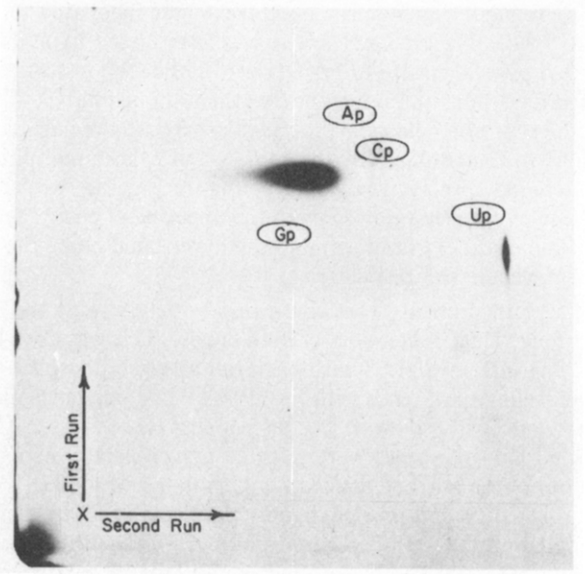

FIGURE 4: Two-dimensional poly(ethylenimine) thin-layer chromatography of a total nuclease digest of intermediate **6** labeled at position b. The spot corresponds to the expected position for CmpUp (Nishimura, 1979).

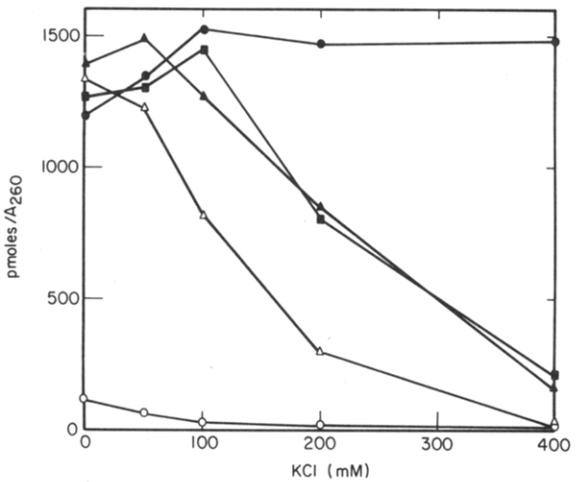

FIGURE 5: Levels of aminoacylation as a function of ionic strength for $tRNA^{Phe}$ (●), $tRNA^{Phe}_{-Y}$ (■), intermediate **2** (△), intermediate **3** with a repaired CCA terminus (○), and $tRNA^{Phe}_{GAAG}$ (▲). Reactions contained 0.2 μM tRNA, 20 μM L-[^{3}H]phenylalanine (5 Ci/mmol), 1 mM ATP, 15 mM $MgCl_2$, 2.5 mM Tris, pH 7.5, 60 mM β-mercaptoethanol, 30 μg/mL BSA, 12 units/mL PRS, and the indicated KCl concentrations. The incubation was carried out at 37 °C for 10 min.

Aminoacylation of Modified tRNAs. With a partially purified preparation of PRS, a preliminary study of the aminoacylation properties of the anticodon-substituted tRNA as well as several of the intermediates in their synthesis was carried out. In Figure 5, the extent of aminoacylation as a function of ionic strength is compared for $tRNA^{Phe}$, $tRNA^{Phe}$

Table I: Kinetic Parameters for Anticodon-Modified tRNA^{Phe}

tRNA[a]	K_m (nM)	V_{max}
tRNA^{Phe}	63	(1.0)
tRNA$^{Phe}_{-Y}$	133	1.4
tRNA$^{Phe}_{1-36,38-76}$ (2)	290	1.8
tRNA$^{Phe}_{1-33,38-76}$ (3A)	*b*	*b*
tRNA$^{Phe}_{GAAG}$ (7)	60	1.4

[a] 3A is intermediate 3 with nucleotides C_{75} and A_{76} added on. tRNA$^{Phe}_{GAAG}$ is 7 with the oligomer GAAG inserted in positions 34–37. [b] Aminoacylation levels were too low to obtain reliable kinetics.

missing Y-37, intermediate **2**, intermediate **3** treated with phosphatase and tRNA nucleotidyltransferase to repair the CCA terminus, and tRNA^{Phe} with GpApApG inserted in the anticodon loop. In this experiment, a large excess of PRS was added, such that additional incubation time did not lead to additional aminoacylation. Suboptimal aminoacylation indicates either an extremely slow forward rate, allowing nonenzymatic deacylation to become an important factor, or an altered equilibrium between the enzymatic acylation and deacylation rates (Bonnet & Ebel, 1972). It is evident from these experiments that the nucleotides in the anticodon loop have a considerable effect on the extent of aminoacylation. At low ionic strength, removal of Y-37 and chain scission have little effect on the level of aminoacylation (Thiebe & Zachau, 1968). However, in agreement with Thiebe et al. (1972), when nucleotides 34–36 are also removed from the anticodon loop, the level of aminoacylation is considerably decreased. Under the reaction conditions in Figure 5, intermediate **3** with a repaired CCA terminus aminoacylates to less than 10% of that observed with tRNA^{Phe}. Finally, when the oligonucleotide GAAG is then inserted into positions 34–37, the high level of aminoacylation is restored. Taken together, these results clearly indicate the importance of the anticodon for optimal aminoacylation of yeast tRNA^{Phe}.

At high ionic strength, the aminoacylation reaction is even more sensitive to anticodon loop modification. Removal of Y-37 causes a substantial decrease in the level of aminoacylation, and chain scission causes an even greater one. Insertion of GpApApG into positions 34–37 does not completely return the level of aminoacylation to that of tRNA^{Phe}. It is possible that at high ionic strength, the presence of Y base is important for optimal aminoacylation. The preparation of tRNA^{Phe} with several different substitutions at position 37 will be required to further explore this point.

In Table I, data on the kinetics of aminoacylation at low ionic strength (25 mM KCl) are presented. Values of K_m and V_{max} were obtained by Lineweaver–Burk analysis of kinetic data obtained at four different tRNA concentrations (0.04–0.35 μM). A 2-fold increase in K_m upon removal of Y-37 and an additional 2-fold increase upon chain scission are observed. No data were obtained for tRNA^{Phe} missing 34–37 because of the low levels of aminoacylation; however, under similar conditions, Thiebe et al. (1972) observed a 17-fold lower V_{max} and a 5-fold higher K_m if no correction for the decreased level of aminoacylation was applied. The insertion of GpApApG into the anticodon loop restores the K_m to nearly the same value as that of tRNA^{Phe}. The value of V_{max} remains somewhat high, perhaps again an indication of the absence of Y base in the tRNA$^{Phe}_{GAAG}$. We can conclude that the kinetic data at low ionic strength substantiate the equilibrium data in identifying the importance of the anticodon loop in the aminoacylation reaction. The nearly normal aminoacylation for tRNA^{Phe} substituted with GpApApG is also good evidence

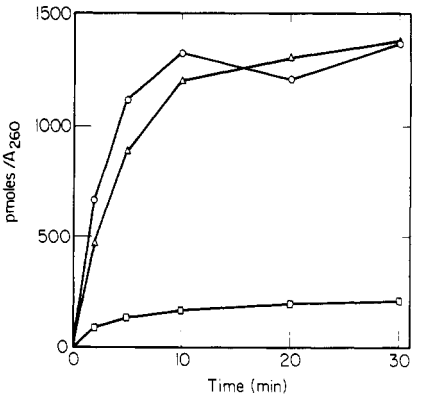

FIGURE 6: Aminoacylation of tRNA^{Phe} (Δ), tRNA$^{Phe}_{GAAG}$ (O), and tRNA$^{Phe}_{A_3C}$ (□). Reactions (50 μL) contained 0.1 μM tRNA, 20 μM L-[^{3}H]phenylalanine (5 Ci/mmol), 1 mM ATP, 25 mM KCl, 15 mM $MgCl_2$, 25 mM Tris (pH 7.5), 60 mM β-mercaptoethanol, 30 μg/mL BSA, and 0.05 unit of yeast PRS and were incubated at 37 °C.

that the multiple steps required for its preparation have not greatly altered its ability to interact specifically with its cognate synthetase.

From the above data, it is not clear what portion of the anticodon loop is involved in the interaction with the synthetase. For example, it is possible that simply the proper positions and number of phosphatases are all that is necessary for full activity. This point was tested by comparing the aminoacylation reactions of tRNA^{Phe}, tRNA^{Phe} with GpApApG substituted in the anticodon, and tRNA^{Phe} with ApApApC substituted in the anticodon. The data shown in Figure 6 indicate that the tRNA with AAAC in the anticodon does not aminoacylate to normal levels at low ionic strength. This suggests that the nucleotide sequence of positions 34–37 is critical for efficient aminoacylation. A more complete kinetic analysis of tRNA$^{Phe}_{AAAC}$ as well as several other anticodon-substituted tRNAs with pure yeast PRS is currently in progress in order to identify the precise functional groups in the anticodon important for the aminoacylation reaction.

Discussion

We have described a procedure for the specific excision of several residues from an internal position in a RNA chain and the subsequent insertion of any oligonucleotide of defined sequence into that position to form a new RNA with a precise sequence modification. The methodology was developed with the anticodon loop of yeast tRNA^{Phe} because of the availability of two convenient cleavage sites, the known three-dimensional structure of the molecule, and the inherent interest of the codon–anticodon interaction. However, it is likely that a similar series of reactions could be carried out for any RNA molecule, provided several criteria could be met. The first of these is the necessity of a specific cleavage procedure for RNA. Limited digestion with nucleases under conditions of low temperature and high ionic strength can often lead to a few specific cleavages. The use of ribonuclease H (Donis-Keller, 1979) also promises to provide specific cleavages at predetermined positions. The second criterion for a successful fragment insertion is that the region of interest should be relatively free of secondary structure. Donors in the RNA ligase reaction are inefficient when part of a secondary structure (Bruce & Uhlenbeck, 1978). Finally, the normal termini of an RNA molecule must be blocked or inaccessible so that RNA ligase cannot act upon them during the oligomer insertion steps. If these criteria are met, the broad substrate specificity of RNA ligase will allow nucleotide substitutions of considerable variety. For example, many sugar- or base-

modified nucleotides are substrates for RNA ligase (Barrio et al., 1978) and thus could be inserted into a RNA molecule.

We have presented evidence that removal of one or more residues from the anticodon loop of $tRNA^{Phe}$ disrupts its interaction with PRS. Removal of Y-37 decreases the level of aminoacylation at high ionic strength and has a discernible effect on the K_m and V_{max} of aminoacylation at low ionic strength. Removal of four nucleotides greatly inhibits aminoacylation under either condition. These data indicated that the anticodon of $tRNA^{Phe}$ binds to the synthetase during the aminoacylation reaction. This view is supported by the observation that Y-37 fluorescence is quenched by PRS (Krauss et al., 1973) and by the detection of a short-lived photocross-link between the anticodon and the protein (Ebel et al., 1979). It has also been shown that the aminoacylation reaction can be inhibited either by the chemical modification of the G-34 with kethoxal (Litt & Greenspan, 1972) or by the addition of an oligomer complementary to the anticodon (Barret et al., 1974).

Preliminary evidence also suggests that the precise sequence of nucleotides in the anticodon has a pronounced effect upon the aminoacylation reaction. Replacement of G_mAAY in positions 34–37 by GAAG has little effect on aminoacylation, but replacement with AAAC leads to a comparatively inactive tRNA. Thus, one or more contacts between the anticodon and the synthetase appear to be sequence specific, identifying the anticodon as part of the "recognition" site of the enzyme. Experiments in progress using purified PRS to obtain aminoacylation kinetics of several different $tRNA^{Phe}$ molecules with single nucleotide changes in the anticodon loop should provide a more precise definition of which nucleotides are important for this interaction.

By comparison of the sequences of 10 tRNAs which mischarged with PRS, Roe et al. (1973) deduced that 10 nucleotides formed an important part of the PRS recognition sites. These residues included A-74 and nine contiguous nucleotides in the D stem and did not include the anticodon. These data do not necessarily disagree with our own, however, since those seven tRNAs which had significantly altered anticodons also showed greatly increased K_m values and decreased V_{max} values with PRS. Thus, the anticodon could be an additional portion of the recognition site of PRS, and only when the correct sequence is present in both positions can normal aminoacylation kinetics be obtained. However, the danger of deducing recognition sites from the aminoacylation properties of tRNAs having very different sequences is clearly brought out by the data of McCutchan et al. (1978). The $tRNA^{Phe}$ from *S. pombe* aminoacylates with PRS with the same kinetics as yeast $tRNA^{Phe}$, even though its sequence is different in 7 of the 10 positions identified by Roe and Dudock. It is interesting to note that the anticodon loop sequence of *S. pombe* $tRNA^{Phe}$ is identical with that of yeast $tRNA^{Phe}$. Possibly the contacts between $tRNA^{Phe}$ and PRS in the highly structured D stem region are more sensitive to more general tertiary structure than precise sequence. In any case, we feel that a more productive way to identify nucleotide-specific "recognition" contacts between PRS and $tRNA^{Phe}$ is by comparing the effects of changes at single positions in a tRNA. When the substrate specificity of RNA ligase and the technology presented in this paper are considered, it should even be possible to compare tRNA molecules which only differ by a few atoms in a single position in the molecule.

Apart from the study of aminoacylation specificity, modified anticodon loops are also expected to have a variety of uses in the study of the details of the codon–anticodon interaction. Toward this end, in collaboration with Dr. J. Atkins and Dr. R. Gesteland, we have recently shown that $tRNA^{Phe}$ with CUAA inserted in positions 34–37 is as active an amber suppressor as yeast Su tRNA in an in vitro protein-synthesizing system. This additionally confirms the activity of these chemically modified tRNA molecules.

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Psoralen-Deoxyribonucleic Acid Photoreaction. Characterization of the Monoaddition Products from 8-Methoxypsoralen and 4,5′,8-Trimethylpsoralen†

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ABSTRACT: The isolation and structural characterization are described of the major monoaddition products formed in the photoreaction of two naturally occurring psoralens, 8-methoxypsoralen and 4,5′,8-trimethylpsoralen, with high molecular weight, double-stranded DNA. Hydrolysis of the psoralen-modified DNA and subsequent chromatography resulted in the isolation of four modified nucleosides from each psoralen. Structural characterization was accomplished by mass spectrometry and ^{1}H NMR analysis. The major products, accounting for 44-52% of the covalently bound psoralen, are two diastereomeric thymidine adducts formed by cycloaddition between the 5,6 double bond of the pyrimidine and the 4′,5′ (furan) double bond of the psoralen. A minor product, less than 2% of the covalently bound psoralen, is a furan-side adduct to deoxyuridine, derived from an initially formed deoxycytidine adduct by hydrolytic deamination. A fourth product is a thymidine adduct where cycloaddition has taken place between the 5,6 double bond of the pyrimidine and the 3,4 (pyrone) double bond of the psoralen. This pyrone-side adduct accounts for 19% of the covalently bound 8-methoxypsoralen but for less than 3% of the covalently bound 4,5′,8-trimethylpsoralen. All of the isolated adducts have cis-syn stereochemistry. The stereochemistry and product distribution of the adducts are determined in part by the constraints imposed by the DNA helix on the geometry of the noncovalent intercalation complex formed by psoralen and DNA prior to irradiation.

Psoralens or furocoumarins are a class of compounds found in a wide variety of plants and fungi and have been used since ancient times as dermal photosensitizing agents for the treatment of various skin pigmentation disorders (Scott et al., 1976). Their photosensitizing activity is dependent upon subsequent exposure to near-ultraviolet light (300-380 nm) and is now known to involve photochemical addition to the DNA of the target tissue (Song & Tapley, 1979). This addition to cellular DNA involves both monoaddition and interstrand cross-linking, as evidenced by denaturation-renaturation kinetics and electron microscopy (Cole, 1970, 1971; Hanson et al., 1976). The ability of psoralens to cross-link double-stranded DNA has attracted interest in their use as probes of nucleic acid structure and function (Wiesehahn et al., 1977; Shen & Hearst, 1977), and they are also under investigation as therapeutic agents for the treatment of psoriasis (Wolff et al., 1977). In addition, their ability to interact efficiently with DNA has drawn attention to their potential as naturally occurring mutagens and carcinogens. The mutagenic activity of compounds such as 8-methoxypsoralen (xanthotoxin) and 5-methoxypsoralen (bergapten) is well established, and reports have appeared documenting the tumorogenic activity of psoralens in a number of species, including man (Ashwood-Smith et al., 1980; Stern et al., 1979; Epstein, 1979).

The photoreaction between psoralens and DNA can be divided into at least three distinct steps: (1) formation of a noncovalent complex with DNA via intercalation of the psoralen between adjacent base pairs; (2) photoreaction between the psoralen and a pyrimidine base to yield a monoadduct; (3) absorption of a second photon to yield an interstrand cross-link. The interstrand cross-links are believed to be largely responsible for the photosensitizing effects of psoralen treatment, although some activity is apparently associated with the monoadducts (Harten et al., 1976). The chemistry of the photoreaction has been studied intensively, but detailed information about the structures of the nucleic acid-psoralen adducts has been notably lacking. The study reported here is concerned with the isolation and identification of the monoaddition products formed from the photobinding to DNA of

† From the Department of Chemistry and Lawrence Berkeley Laboratory, University of California, Berkeley, California 94720. *Received August 3, 1981.* This research was supported in part by the Office of Environmental Research and Development, Biomedical and Environmental Research Division, Department of Energy, under Contract No. W-7405-ENG-48, and the National Institute of General Medical Sciences (Grant GM-25151). NMR studies were carried out at the University of California, Davis, NMR Facility under the auspices of National Science Foundation Grant CHE 79-04832 or at the Stanford Magnetic Resonance Laboratory under the auspices of National Science Foundation and National Institutes of Health Grants GP-263633 and RR-00711. Mass spectrometry studies were carried out at the University of California, Berkeley, Bioorganic, Biomedical Mass Spectrometry Resource supported by National Institutes of Health Grant RR-00719, Dr. A. L. Burlingame, Director.

0006-2960/82/0421-0861$01.25/0