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Aminoacylation of Anticodon Loop Substituted Yeast Tyrosine Transfer RNA†

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ABSTRACT: A procedure for replacing residues 33–35 in the anticodon loop of yeast $tRNA^{Tyr}$ with any desired oligonucleotide has been developed. The three residues were removed by partial ribonuclease A digestion. An oligonucleotide was inserted into the gap in four steps by using RNA ligase, polynucleotide kinase, and *pseT* 1 polynucleotide kinase. The rate of aminoacylation of anticodon loop substituted $tRNA^{Tyr}$ by yeast tyrosyl-tRNA synthetase was found to depend upon the sequence of the oligonucleotide inserted. This suggests that the nucleotides in the anticodon loop of yeast $tRNA^{Tyr}$ are required for optimal aminoacylation. In addition, $tRNA^{Tyr}$ modified to have a phenylalanine anticodon was shown to be misacylated by yeast phenylalanyl-tRNA synthetase at a rate at least 10 times faster than unmodified $tRNA^{Tyr}$. Thus, the anticodon is used by phenylalanyl-tRNA synthetase to distinguish between tRNAs.

When a tRNA reacts with its cognate aminoacyl-tRNA synthetase, the anticodon loop is often in contact with the surface of the enzyme. Kisselev (1983) summarizes a variety of experiments indicating the involvement of the anticodon for eight different *Escherichia coli* and five different yeast tRNA synthetases. In several instances, it is clear that functional groups of anticodon nucleotides are important in the interaction. If anticodon residues 34 or 35 of *E. coli* $tRNA_f^{Met}$ are chemically modified (Schulman & Pelka, 1977) or altered in their sequence (Schulman et al., 1983a), the rate of aminoacylation is drastically reduced. Mutations in the anticodons of *E. coli* $tRNA^{Gly}$ (Carbon & Squires, 1971) and $tRNA^{Trp}$ (Yarus et al., 1977) also have been shown to alter their rate of aminoacylation. These experiments support the view that anticodon loop residues could be used by aminoacyl-tRNA synthetases to distinguish between different tRNAs (Kisselev & Frolova, 1964).

In this work, we describe an enzymatic procedure to alter the sequence of residues 33–35 of yeast $tRNA^{Tyr}$ to any desired sequence. The procedure closely resembles the protocol previously developed for substituting nucleotides 34–37 in yeast $tRNA^{Phe}$ (Bruce & Uhlenbeck, 1982a) although different anticodon residues are changed. We have used these anticodon-substituted $tRNAs^{Tyr}$ to demonstrate that yeast tyrosyl-tRNA synthetase also requires the correct anticodon sequence for optimal rates of aminoacylation. In addition the substitution of Ψ-35 with an A produces a $tRNA^{Tyr}$ with a phenylalanine anticodon. Since it has been shown previously that yeast phenylalanyl-tRNA synthetase requires the correct anticodon sequence in $tRNA^{Phe}$ to give an optimal rate of aminoacylation (Bruce & Uhlenbeck, 1982b), the misacylation of the A-35 $tRNA^{Tyr}$ with phenylalanyl-tRNA synthetase was investigated. We show that the substitution results in a substantial increase in misacylation when compared to $tRNA^{Tyr}$. These results clearly indicate that a contact at A-35 is important in the interaction of the tRNA with phenylalanyl-tRNA synthetase.

Materials and Methods

Enzymes. RNA ligase (Moseman-McCoy et al., 1979) and polynucleotide kinase (Cameron & Uhlenbeck, 1977) were purified from T4-infected *E. coli*. Polynucleotide kinase lacking the 3′-phosphatase activity was purified from *pseT* 1 T4 infected *E. coli* (Soltis & Uhlenbeck, 1982). Primer-dependent polynucleotide phosphorylase was purified from *Micrococcus luteus* (Klee, 1971). Yeast tRNA nucleotidyltransferase was a gift from P. Sigler. Homogeneous yeast phenylalanyl-tRNA synthetase was a gift from P. Remy. Yeast tyrosyl-tRNA synthetase was purified to a specific activity of 200 units/mg by using the first three steps of the procedure of Faulhammer & Cramer (1977). Ribonucleases T_1, T_2, P_1, and Cl_3 were purchased from Boehringer-Mann-

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heim. Ribonuclease A was purchased from Sigma Chemical Co. Ribonuclease Phy M was a gift from H. Donis-Keller.

Nucleotides and tRNAs. UpG, GDP, and pseudouridine were purchased from Sigma. [γ-^{32}P]ATP was prepared from [^{32}P]orthophosphate by the method of Johnson & Walseth (1979), and [5′-^{32}P]pCp was prepared as described by England et al. (1980). UpGpA and UpGpU were prepared from polynucleotide phosphorylase (Thach & Doty, 1965). ApApC was prepared from a ribonuclease A digest of poly(AC) (Bruce & Uhlenbeck, 1978). Purified yeast tRNA^{Phe} was purchased from Boehringer-Mannheim. Yeast tRNA^{Tyr} was purified as described by Maxwell et al. (1968).

Synthesis of UGΨ. The trinucleoside diphosphate UpGpΨ was synthesized with ribonuclease T_1 as described by Mohr & Thach (1969). UpGp was prepared in a 1-mL reaction containing 8.2 mM UpG, 21 mM GDP, 0.4 M NaCl, 10 mM $MgCl_2$, 0.2 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 8.2, 100 μg/mL ribonuclease T_1, and 7 units/mL polynucleotide phosphorylase. After 4 days at 37 °C, UpGp was purified by high-pressure liquid chromatography on a 4.6 × 100 mm C-18 column using a 10-min linear gradient from 1% CH_3CN to 4.2% CH_3CN (McFarland & Borer, 1979). UpGp (6.2 μmol, 73%) was absorbed onto Whatman 3MM paper, desalted by washing with absolute ethanol, and eluted with water.

UpGp was converted to UpG>p in a 0.5-mL reaction containing 12 mM UpGp, 100 mM 2-(*N*-morpholino)-ethanesulfonic acid (MES), pH 5.5, and 30 mM *N*-ethyl-*N*′-[3-(dimethylamino)propyl]carbodiimide hydrochloride (Naylor & Gilham, 1966). After 1 h at 25 °C, the UpG>p was purified by descending chromatography on Whatman 3MM paper using a solvent of 40% 1 M ammonium acetate and 60% ethanol. After desalting and elution, 4.3 μmol (70%) UpG>p was recovered. The identity of UpG>p was confirmed by showing it to be resistant to bacterial alkaline phosphatase and to be converted to UpGp upon treatment with ribonuclease T_1.

UpGpΨ was prepared in a 0.4-mL reaction containing 8 mM UpG>p, 175 mM pseudouridine, 50 mM Tris-HCl, pH 7.5, and 2 μg/mL ribonuclease T_1. After 20 min at 0 °C, the reaction was quenched by the addition of 0.8 mL of 50 mM dithiothreitol and 7.5 M NH_4OH (Mohr & Thach, 1969). The UpGpΨ (0.9 μmol, 28%) was purified by descending chromatography on Whatman 3MM paper with a solvent of 70:10:20 by volume of 95% ethanol, concentrated NH_4OH, and water, respectively. The putative UpGpΨ was identified by labeling the 3′ terminus with RNA ligase and [5′-^{32}P]pCp or the 5′ terminus with polynucleotide kinase and [γ-^{32}P]ATP, digesting the labeled oligomers with ribonuclease T_2, and analyzing the radiolabeled nucleotides by two-dimensional thin-layer chromatography (Nishimura, 1979). The appearance of [5′-^{32}P]pUp as a result of the 5′ labeling and [^{32}P]Ψp as a result of the 3′ labeling confirmed the identity of UpGpΨ.

Synthesis of Anticodon Loop Substituted tRNA^{Tyr}. The intermediates in the six-step protocol for substituting residues 33–35 in yeast tRNA^{Tyr} are shown in Figure 1. The first step was a partial ribonuclease A digestion of tRNA^{Tyr}. The 6.9-mL reaction contained 15.8 μM tRNA^{Tyr}, 100 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 200 mM KCl, and 20 μg/mL ribonuclease A. After 2 h at 0 °C, the reaction was terminated by adding 108 μL of diethyl pyrocarbonate, vortexing for 30 s and immediately extracting twice with phenol. The combined tRNA half-molecules (**2**) were recovered by ethanol precipitation. The treatment with diethyl pyrocarbonate was essential for terminating the nuclease digestion reaction. If it was omitted, further digestion occurred during the phenol extraction. Control experiments demonstrated that treating tRNA^{Tyr} with diethyl pyrocarbonate in a similar fashion did not alter the rate or extent of aminoacylation.

The second step involved the phosphorylation of the 5′ end of the 3′ half-molecule. The 1.5-mL reaction contained 23 μM **2**, 230 μM ATP, and 72 units/mL *pseT* 1 polynucleotide kinase in ligase buffer. Ligase buffer contained 50 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes), pH 8.3, 20 mM $MgCl_2$, 3.3 mM dithiothreitol, and 10 μg/mL bovine serum albumin. In certain cases, [γ-^{32}P]ATP (110 μCi/mmol) was introduced at this step. The reaction was incubated for 2 h at 37 °C and terminated by heating to 65 °C for 4 min. Intermediate **3** was recovered by ethanol precipitation.

The addition of the trimer to the 3′ half-molecule was carried out in a 0.7-mL reaction containing 10 μM **3**, 30 μM trimer, 100 μM ATP, and 59 μg/mL RNA ligase in ligase buffer. After 4 h at 16 °C, the reaction was heated to 65 °C for 3 min, and intermediate **4** was recovered by ethanol precipitation.

The phosphorylation of the 5′ terminus of the 3′ half-molecule was carried out in a 0.65-mL reaction containing 11 μM **4**, 30 μM ATP, and 90 units/mL polynucleotide kinase in ligase buffer adjusted to pH 6.8. In certain instances a [^{32}P]phosphate was introduced in this step by including [γ-^{32}P]ATP (110 μCi/mmol). After 2 h at 37 °C, the reaction was stopped by heating at 65 °C for 4 min.

The joining of the half-molecules was accomplished by adding RNA ligase to the previous reaction to a final concentration of 14.5 μg/mL. After 3 h at 16 °C, intermediate **6** was ethanol precipitated.

The 3′ terminus was repaired in a 0.65-mL reaction containing 10 μM **6**, 200 μM CTP, 500 μM ATP, 10 mM Tris-HCl, pH 8.8, 10 mM $MgCl_2$, 1 mM dithiothreitol, and enough tRNA nucleotidyltransferase to repair the 3′ terminus in 2 h at 25 °C. After ethanol precipitation of the anticodon-substituted tRNA, it was resuspended in 80 μL of 5 M urea, 0.2% (w/v) bromophenol blue, and 0.3% (w/v) xylene cyanol and applied to a 30 mm wide well of a 430 × 150 × 0.75 mm 20% polyacrylamide sequencing gel (Donis-Keller et al., 1977). After electrophoresis at 1000 V for 34 h, the tRNA band was purified from the gel as described previously (Bruce & Uhlenbeck, 1982a).

Aminoacylation. Reactions of 30 or 60 μL contained the indicated concentration of tRNA between 30 nM and 2.5 μM, 10 μM [^{3}H]tyrosine (51 Ci/mmol) or [^{3}H]phenylalanine (28 Ci/mmol), 2 mM ATP, 30 mM Hepes, pH 7.5, 55 mM KCl, 15 mM $MgCl_2$, 4 mM dithiothreitol, and the indicated concentration of tyrosyl-tRNA synthetase or phenylalanyl-tRNA synthetase. Incubation was at 37 °C, and 5- or 10-μL aliquots were withdrawn at the indicated times, precipitated with trichloroacetic acid, filtered on nitrocellulose filters, and counted in a liquid scintillation counter. To obtain the K_m and V_{max} values in Table I, initial rates were determined in the same buffer at pH 7 by using tRNA concentrations ranging between 0.2 and 6.5 μM for tRNA^{Tyr} and tRNA^{Tyr} (UGA) and between 0.030 and 0.5 μM for tRNA^{Phe}. The data were analyzed by using Eadie–Hofstee plots. The "accurate" aminoacylation buffer contained 1 mM ATP, 1.1 mM $MgCl_2$, 100 μM spermine, 10 μM [^{3}H]phenylalanine (28 Ci/mmol), 30 mM Hepes, pH 7.5, 55 mM KCl, and 4 mM dithiothreitol.

Results

Construction of Anticodon Loop Substituted tRNA^{Tyr}. The six-step procedure for enzymatically replacing nucleotides

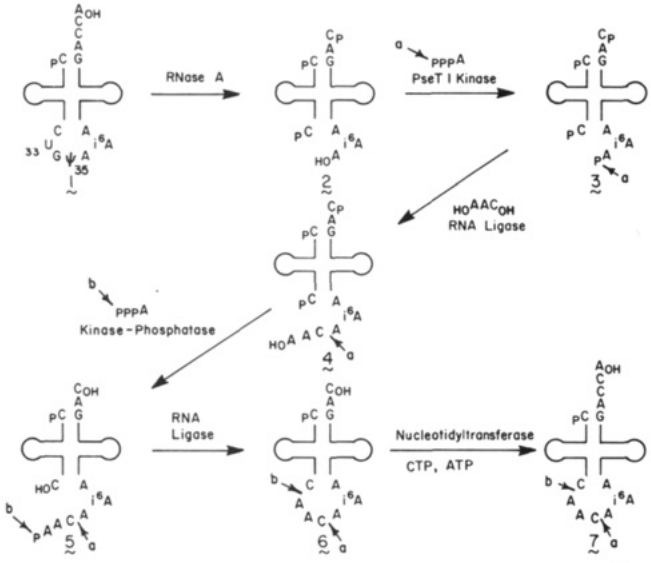

FIGURE 1: Steps in the substitution of residues 33–35 in yeast $tRNA^{Tyr}$. An internal ^{32}P can be introduced at position a or b by including [γ-^{32}P]ATP in the polynucleotide kinase reaction.

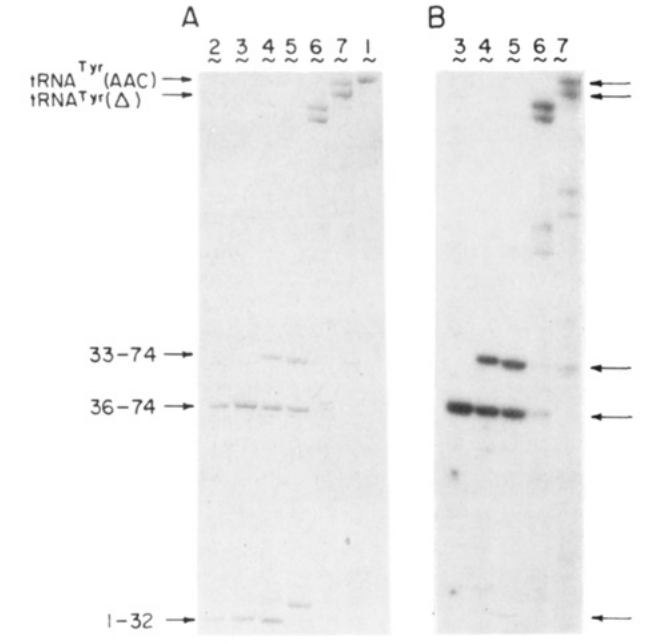

FIGURE 2: (A) Denaturing 20% polyacrylamide gel of intermediates **1**–**7** stained with "Stains All". (B) Autoradiograph of intermediates **3**–**7** of (A) which contains ^{32}P in the a-position.

33–35 in the anticodon loop of yeast $tRNA^{Tyr}$ by the oligonucleotide ApApC is shown in Figure 1. The products of each step of the procedure were analyzed by high-resolution denaturing polyacrylamide gel electrophoresis. A gel of the stained reaction products is shown in Figure 2A, and an autoradiogram of the same gel is shown in Figure 2B. Intermediates **3**–**7** were ^{32}P labeled as a result of introducing [γ-^{32}P]ATP in the second step.

The first step involved partial digestion of yeast $tRNA^{Tyr}$ with pancreatic ribonuclease A at 0 °C and high sodium and magnesium ion concentration. Presumably due to the stable tertiary structure of the tRNA under the conditions, ribonuclease digestion led to rapid accumulat of two fragments each about 30 nucleotides long and oni idual formation of smaller fragments (lane 2, Figure 2A) le, if any, intact $tRNA^{Tyr}$ was left after digestion. Each i ent was excised from the gel and, after dephosphorylati ith bacterial alkaline phosphatase, was either 5′ end labeled with [γ-^{32}P]ATP and polynucleotide kinase or 3′ end labeled with [5′-^{32}P]pCp and RNA ligase. The 3′- and 5′-end-labeled half-molecules were individually purified on a similar gel, eluted, and digested with ribonuclease T_2. Analysis of the products of the digestion by two-dimensional thin-layer chromatography indicated that the lower band had C at its 5′ terminus and C at its 3′ terminus, while the upper band had A at its 5′ terminus and C at its 3′ terminus (data not shown). The length of the two $tRNA^{Tyr}$ fragments was deduced by comparing their electrophoretic mobility with $tRNA^{Phe}$ half-molecules of known length. Together these data indicate that the lower band is the 5′ half-molecule containing residues 1–32 and the upper band is the 3′ half-molecule containing residues 36–74. Thus, the preferential cleavage sites of $tRNA^{Tyr}$ by pancreatic ribonuclease A were after positions 32 and 35 in the anticodon and after position 74 at the 3′ terminus, resulting in two annealed half-molecules (intermediate **2**).

In the second step the 3′ half-molecule in **2** was phosphorylated with *pseT* 1 polynucleotide kinase. Since this mutant enzyme lacks the inherent 3′-phosphatase activity of polynucleotide kinase, A-36 can be 5′ phosphorylated without dephosphorylating the 3′-phosphates at C-33 and C-74. These 3′-phosphates were needed as blocking groups to protect the 3′ terminus in the subsequent RNA ligase reaction. The result of such a phosphorylation reaction using [γ-^{32}P]ATP is shown in lane 3 of Figure 2B. Since the 5′ half-molecule already had a 5′-phosphate, radioactivity was incorporated onto the 3′ half-molecule only. Interestingly, no shift in electrophoretic mobility occurred as a result of phosphorylation, thereby emphasizing the lack of predictability of the mobility of oligonucleotides on RNA sequencing gels. It was therefore necessary to determine the yield of this step indirectly in a separate reaction. This reaction was identical except that it included an equal amount of 3′ half-molecule of yeast $tRNA^{Phe}$ (residues 34–74) and [γ-^{32}P]ATP. By use of conditions where the $tRNA^{Phe}$ fragment was totally phosphorylated as judged by its mobility shift, the $tRNA^{Tyr}$ fragment had incorporated an identical amount of radioactivity. Thus, the yield of intermediate **3** from **2** was estimated to be greater than 90%.

The ligation of the trimer ApApC onto the 3′ half-molecule was the third step in the substitution procedure. The addition of the trimer substantially retards the mobility of the 3′ half-molecule as shown in lane 4 of Figure 2. No addition of the trimer to the 5′-phosphate of the 5′ half-molecule was observed, which is consistent with the lack of reactivity of the 5′ terminus of intact tRNA (Bruce & Uhlenbeck, 1978). The yield of the RNA ligase reaction was estimated to be about 50% by comparing the radioactivity in the two 3′ half-bands. Depending upon the identity of the trimer acceptor, the yield at this step varied from about 10% to 60%. These yields are considerably lower than the greater than 90% yields reported for the addition of an oligonucleotide acceptor to the 3′ half-molecule (residues 38–74) of yeast $tRNA^{Phe}$ under very similar reaction conditions (Bruce & Uhlenbeck, 1982a). The reason for this difference is not clear. Competition experiments in which $tRNA^{Phe}$ and $tRNA^{Tyr}$ half-molecules were mixed and the reaction yields with a single acceptor compared have shown that the 3′ half of $tRNA^{Tyr}$ was a much less effective donor.

Despite an extensive search for alternate reaction conditions, quantitative yields could not be obtained in this step. When higher RNA ligase concentrations or longer incubation times are used, a new band accumulates which migrates slightly faster than the 5′-phosphorylated 3′ half-molecule. End la-

beling experiments identified this molecule as residues 38–74 having a 5′-hydroxyl and a 3′-phosphate which presumably arises as a result of degradation of the 3′ half-molecule. Since this fragment often accounted for 5–15% of the total 3′ half-molecule in successful reactions, the addition of more RNA ligase will not give higher yields. Attempts to suppress this presumed nuclease activity have not been successful.

The fourth step was the simultaneous removal of the 3′-terminal phosphates from both half-molecules and the phosphorylation of newly made 5′ terminus of the 3′ half-molecule. Both reactions are catalyzed by T4 polynucleotide kinase–3′-phosphatase. This step is identical with the one carried out in the $tRNA^{Phe}$ substitution procedure (Bruce & Uhlenbeck, 1982a). As shown in lane 5 of Figure 2, the 3′ half-molecule has no mobility shift since it both gains a 5′-phosphate and loses a 3′-phosphate. However, the 5′ half-molecule shows a distinct decrease in mobility due to the loss of its 3′ phoshate. Although nonradioactive ATP was used for this step in Figure 2, a radioactive phosphate could have been incorporated in this position as well. The yield of this step was estimated to be greater than 90%.

In the fifth step a low concentration of RNA ligase was added to the reaction mixture of the previous step after the polynucleotide kinase had been inactivated by heating. As shown in lane 6 of Figure 2, joining of the annealed half-molecules to form tRNA sized products occurred very efficiently. However, since both 3′ half-fragments were capable of reacting with the 5′ half, two product bands were obtained. The upper band has the anticodon loop sequence replaced as desired while the lower band is a tRNA in which the anticodon loop had resealed in the absence of the trinucleotide. While the extent of formation of the lower band could be reduced by lowering the reaction temperature to 16 °C, it was not possible to completely suppress the undesired sealing reaction.

The final step in the construction was to repair the 3′ terminus of the tRNA by using tRNA nucleotidyltransferase. As shown in lane 7 of Figure 2, both the bands in lane 6 are shifted to a slower mobility as a result of the addition of two residues to their 3′ terminus. The upper band of the doublet now has the same mobility as intact $tRNA^{Tyr}$ in lane 1 and is the anticodon loop substituted $tRNA^{Tyr}$ (AAC). The lower band of the doublet is a $tRNA^{Tyr}$ missing residues 33–35 and thus has an anticodon loop of four residues. It will be called $tRNA^{Tyr}$ (Δ). Preparative scale reactions of each anticodon loop substituted tRNAs were purified by gel electrophoresis. In some reactions a large amount of $tRNA^{Tyr}$ (Δ) was formed, and it was difficult to obtain the substituted $tRNA^{Tyr}$ entirely free of $tRNA^{Tyr}$ (Δ). The overall recovered yield of the reaction varied from 6% to 12% depending upon the trimer sequence inserted.

Product Identification. An enzymatic RNA sequence determination of $tRNA^{Tyr}$ (AAC) labeled at the 3′ terminus with [5′-^{32}P]pCp is shown in Figure 3. The presence of the appropriate ribonuclease Cl_3 and U_2 cleavages in the anticodon region clearly confirms the insertion of AAC in positions 33–35. The absence of a ribonuclease T_1 cleavage at position 34 indicates that the product is not significantly contaminated with $tRNA^{Tyr}$ since the guanosine at 34 is especially sensitive to T_1 hydrolysis in that tRNA.

In order to better determine the purity of the product, two independent syntheses of $tRNA^{Tyr}$ (AAC) were carried out with [γ-^{32}P]ATP at either the first polynucleotide kinase step (indicated as the a position in Figure 1) or the second polynucleotide kinase step (indicated as the b position in Figure 1). Each radiolabeled tRNA was purified and then digested

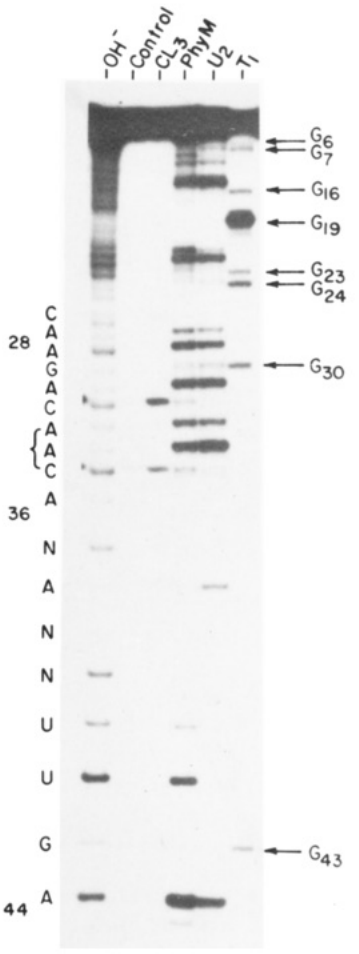

FIGURE 3: Enzymatic RNA sequence determination of 3′-end-labeled $tRNA^{Tyr}$ (AAC). The nucleotide sequence of the left was deduced from the combined partial enzymatic digestion patterns. The bracket indicates the position where ApApC was substituted into $tRNA^{Tyr}$. Guanosine positions are labeled and numbered on the right.

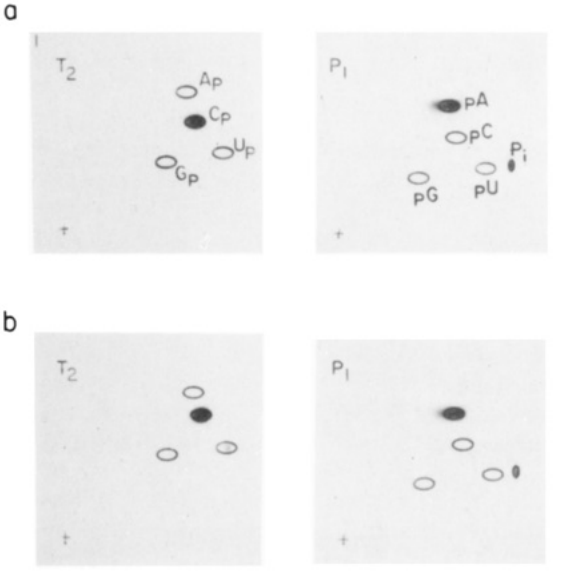

FIGURE 4: Nucleotide analysis of hydrolyzed $tRNA^{Tyr}$ (AAC) with a ^{32}P in position a or position b (see Fig. 1). Hydrolysis was carried out with either RNase T_2 (left) or P_1 (right).

with ribonuclease T_2 to give nucleoside 3′-monophosphates or nuclease P_1 to give nucleoside 5′-monophosphates. The products of the digestion were analyzed by two-dimensional thin-layer chromatography, and the radiolabeled nucleotides

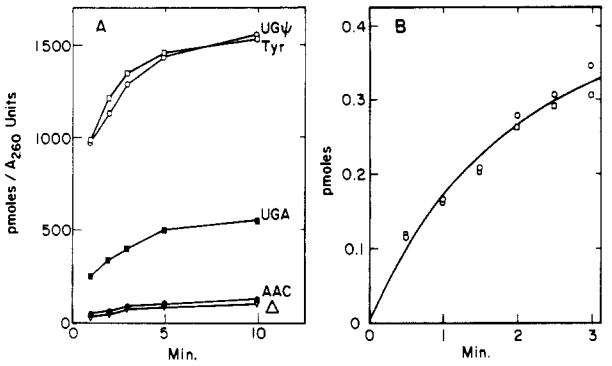

FIGURE 5: (A) Aminoacylation of $tRNA^{Tyr}$ (□), $tRNA^{Tyr}$ (UGΨ) (○), $tRNA^{Tyr}$ (UGA) (■), $tRNA^{Tyr}$ (AAC) (●), and $tRNA^{Tyr}$ (Δ) (▽). Reactions contained 300 nM tRNA and 0.7 unit/mL tyrosyl-tRNA synthetase. (B) Rate of aminoacylation for $tRNA^{Tyr}$ (○) and $tRNA^{Tyr}$ (UGΨ) (□). Reactions contained 150 nM tRNA and 0.09 unit/mL tyrosyl-tRNA synthetase.

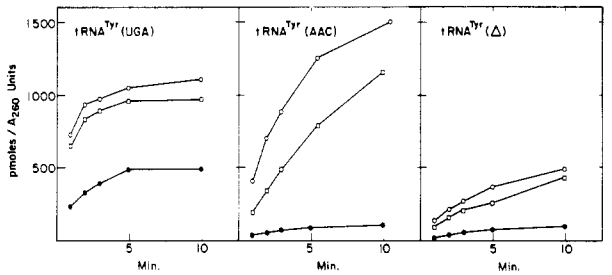

FIGURE 6: Aminoacylation of $tRNA^{Tyr}$ (UGA), $tRNA^{Tyr}$ (AAC), and $tRNA^{Tyr}$ (Δ) with 13.6 (○), 3.4 (□), or 0.7 units/mL (●) of tyrosyl-tRNA synthetase. All reactions contain 300 nM tRNA.

were localized by autoradiography. As shown in Figure 4, the $tRNA^{Tyr}$ (AAC) labeled at either the a or the b position gave [^{32}P]-3′-CMP and [^{32}P]-5′-AMP as expected. Thus, the substituted $tRNA^{Tyr}$ (AAC) was not contaminated by other species that could have arisen during the protocol. Several of the other substituted tRNAs were tested in a similar fashion to confirm the identity of the insertion and the purity of the product.

Aminoacylation of Anticodon Loop Substituted $tRNAs^{Tyr}$. The kinetics of aminoacylation of three anticodon loop substituted $tRNAs^{Tyr}$ and $tRNA^{tyr}$ (Δ), with yeast tyrosyl-tRNA synthetase are compared with $tRNA^{Tyr}$ in Figure 5. $tRNA^{Tyr}$ (UGΨ), in which the same residues that were excised were reintroduced, aminoacylated to the same level (panel A) and at the same initial rate (panel B) as $tRNA^{Tyr}$. This control experiment clearly shows that the anticodon loop substitution protocol does not alter other portions of the RNA molecule which might affect aminoacylation.

The other anticodon loop substituted $tRNAs^{Tyr}$ were less active than $tRNA^{Tyr}$. At 0.7 unit/mL tyrosyl-tRNA synthetase, $tRNA^{Tyr}$ (UGA) aminoacylated to an intermediate extent whereas $tRNA^{Tyr}$ (AAC) and $tRNA^{Tyr}$ (Δ) did not aminoacylate appreciably. Since yeast tyrosyl-tRNA synthetase does not enzymatically deacylate Tyr-$tRNA^{Tyr}$ (von der Haar & Cramer, 1976), these lower levels of aminoacylation are a consequence of a slow forward rate that cannot offset the spontaneous deacylation of the charged tRNA. This is supported by the fact that, at higher enzyme concentrations, the level of aminoacylation increased to much higher levels for $tRNA^{Tyr}$ (AAC) and $tRNA^{Tyr}$ (UGA) (Figure 6). Although a more quantitative kinetic study must be done, it is clear from these data that the correct anticodon sequence is essential for an optimal rate of aminoacylation with tyrosyl-tRNA synthetase.

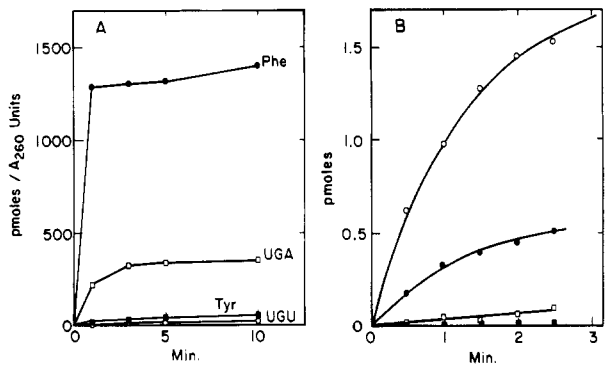

FIGURE 7: (A) Aminoacylation of $tRNA^{Phe}$ (○), $tRNA^{Tyr}$ (UGA) (□), $tRNA^{Tyr}$ (■), and $tRNA^{Tyr}$ (UGU) (○) with phenylalanyl-tRNA synthetase. Reactions contained 675 nM tRNA and 154 units/mL phenylalanyl-tRNA synthetase. (B) Rates of aminoacylation of $tRNA^{Tyr}$ (squares) and $tRNA^{Tyr}$ (UGA) (circles) with phenylalanyl-tRNA synthetase. Reactions in normal aminoacylation buffer are represented as open figures. Reactions done in buffer containing spermine are represented as solid figures. All reactions contained 2 μM tRNA and 154 units/mL phenylalanyl-tRNA synthetase.

Table I: Kinetic Parameters for Aminoacylation with Phenylalanyl-tRNA Synthetase

	K_m (nM)	V_{max}
$tRNA^{Phe}$	93	(1.0)
$tRNA^{Tyr}$ (UGA)	1200	0.015
$tRNA^{Tyr}$	2500	0.002

The misacylation of $tRNA^{Tyr}$ and $tRNA^{Tyr}$ (UGA) with purified yeast phenylalanyl-tRNA synthetase are shown in Figure 7. At an enzyme concentration about 50 times the amount required to saturate $tRNA^{Phe}$, $tRNA^{Tyr}$ (UGA) aminoacylated to about 26% of its theoretical level. This misacylation is clearly a consequence of the A at position 35 since $tRNA^{Tyr}$ and $tRNA^{Tyr}$ (UGU) do not misacylate appreciably under these conditions (Figure 7A). A comparison of the initial rate of the reactions (Figure 7B) showed a 12.5-fold increase in misacylation rate as a result of the substitution of the Ψ in $tRNA^{Tyr}$ by an A in $tRNA^{Tyr}$ (UGA). The initial rates of misacylation of the same two tRNAs were also compared in a buffer containing spermine which has been shown to substantially increase the accuracy of aminoacylation (Loftfield et al., 1981). Although the misacylation rates of both $tRNA^{Tyr}$ and $tRNA^{Tyr}$ (UGA) are a good deal lower as expected, the latter is still at least 10-fold greater (Figure 7B). These results indicate that the stimulation of misacylation by the Ψ to A change is not simply a consequence of using a particular buffer.

In Table I, the kinetic parameters of $tRNA^{Phe}$, $tRNA^{Tyr}$, and $tRNA^{Tyr}$ (UGA) with phenylalanyl-tRNA synthetase are compared in the standard buffer. As a result of the Ψ to A substitution, the K_m is improved about 2-fold and the V_{max} about 7-fold. However, $tRNA^{Tyr}$ (UGA) aminoacylates with a 13-fold greater K_m and a 70-fold smaller V_{max} than $tRNA^{Phe}$, suggesting that other contacts are needed to obtain an optimal rate with phenylalanyl-tRNA synthetase.

Discussion

The protocol we describe for substituting nucleotides in the anticodon loop of yeast $tRNA^{Tyr}$ closely resembles the protocol previously developed in this laboratory for yeast $tRNA^{Phe}$ (Bruce & Uhlenbeck, 1982a). In both cases the sensitivity of the anticodon loop to nuclease digestion was exploited to create a gapped tRNA that was used as a donor in an RNA ligase reaction. Since the 5′ terminus of the 5′ half-molecule

is not an effective donor, a short oligomer acceptor is preferentially added onto the 3′ half-molecule. The annealed 5′ half then served to protect the 3′ half from degradation and was present for the subsequent joining reaction. The $tRNA^{Tyr}$ protocol substitutes positions 33–35 in the anticodon and thus, unlike $tRNA^{Phe}$, does not disturb the hypermodified purine at position 37 and permits alteration of the constant uridine at position 33 (Bare et al., 1983). Although the protocol gave substantial amounts of anticodon-substituted $tRNA^{Tyr}$, the overall yields were much less than those obtained for $tRNA^{Phe}$. The difference in yield was primarily the result of incomplete addition of the trinucleotide onto the 3′ half-molecule. Despite many attempts to increase the yield at this step, it appears that, perhaps due to the isopentenyl-A at position 37, the 3′ half-molecule of $tRNA^{Tyr}$ is a very inefficient RNA ligase donor. One consequence of the poor yield at this early step is that a $tRNA^{Tyr}$ missing part of the anticodon loop is produced which complicates the purification of the substituted tRNA.

Although the data are incomplete, it is clear that substitution of nucleotides in the anticodon loop of yeast $tRNA^{Tyr}$ alters the kinetics of the aminoacylation reaction with the cognate tyrosyl-tRNA synthetase. In the buffer conditions employed, about a 10-fold reduction in the forward reaction rate was observed. This reduction is similar to that observed with yeast phenylalanine synthetase when nucleotides are substituted in the anticodon of yeast $tRNA^{Phe}$ (Bruce & Uhlenbeck, 1982b) and with *E. coli* tryptophanyl-tRNA synthetase when C-35 in $tRNA^{Trp}$ is changed to a U (Yarus et al., 1977). Much greater effects are seen when anticodon nucleotides are substituted in *E. coli* $tRNA^{fMet}$ (Schulman et al., 1983b) and $tRNA^{Gly}$ (Carbon & Squires, 1971). Since each position in the $tRNA^{Tyr}$ anticodon has not been tested independently, it is not clear whether only one or several residues in the anticodon loop are important for optimal aminoacylation. The lower rate observed for $tRNA^{Tyr}$ (UGA) indicates that position 35 appears important for aminoacylation with yeast tyrosyl-tRNA synthetase. This result is interesting in light of the observation that a Ψ to U change at position 35 reduces the suppressor activity of *SUP6* tRNA derived from $tRNA^{Tyr}$ (Johnson & Abelson, 1983). It is possible that the U-containing tRNA may aminoacylate at a reduced rate and thereby explain its poor suppressor activity. This possiblity is also of interest in regard to position 34. Although it is generally supposed that the G to C change at this position required to make an amber suppressor tRNA does not alter the aminoacylation rate, data on the aminoacylation kinetics of suppressor tRNAs are incomplete (Abelson et al., 1970). A systematic study on each position in $tRNA^{Tyr}$ will be required to settle these points. However, it is already clear that like most synthetases that have been examined, tyrosyl-tRNA synthetase requires the correct anticodon sequence of its cognate tRNA for optimal aminacylation.

The rate of misacylation of $tRNA^{Tyr}$ by phenylalanyl-tRNA synthetase is increased at least 10-fold by the substitution of the pseudouridine at position 35 by an adenosine. This result appears to be true even in a buffer known to give a very low rate of misacylation. Since substituting the adenosine at position 35 in $tRNA^{Phe}$ to any other nucleotide causes a decrease in the rate of aminoacylation (Bruce & Uhlenbeck, 1982b), it is clear that an A at the central codon position aids the phenylalanyl-tRNA synthetase reaction. Although more complicated interpretations of these two facts could be made, it is simplest to conclude that functional groups on the adenosine at that position interact specifically with the protein. Thus, A-35 is one of several positions on $tRNA^{Phe}$ where the nucleotide sequence is used directly by phenylalanyl-tRNA synthetase to discriminate between different tRNAs. A similar situation is observed with *E. coli* Su^{+7} tRNA where substitution of C-35 of $tRNA^{Trp}$ by a U greatly increases the rate of misacylation by glutamyl-tRNA synthetase (Yarus et al., 1977). Since $tRNA^{Glu}$ has a U at position 35, it suggests that the glutamyl-tRNA synthetase uses the nucleotide sequence at this position as well. Lestienne (1978) has suggested on the basis of tRNA sequence comparisons that many synthetases could use position 35 for discrimination among tRNAs.

Among tRNAs with phenylalanine anticodons, $tRNA^{Tyr}$ (UGA) is a very poor substrate for yeast phenylalanine synthetase. The kinetic constants for the aminoacylation of wheat germ $tRNA^{Phe}$ and *S. pombe* $tRNA^{Phe}$ are within a factor of 2 of those obtained with the homologous *Saccharomyces cerevisiae* $tRNA^{Phe}$ (Roe et al., 1973b; McCutchan et al., 1978). Even *E. coli* $tRNA^{Phe}$ aminoacylates quite well, especially after G-10 has been converted to m^2G (Roe et al., 1973a). In contrast, both the K_m and the V_{max} of $tRNA^{Tyr}$ (UGA) are reduced by about an order of magnitude. It is tempting to speculate that $tRNA^{Tyr}$ (UGA) is missing one or more essential residues necessary for correct interaction with the synthetase and to attempt to identify these residues by comparing the sequence of $tRNA^{Tyr}$ (UGA) with the $tRNA^{Phe}$ sequences. However, it was this general approach that led Roe et al. (1973a) to the incorrect conclusion that the anticodon was not involved in synthetase recognition. The difficulty in evaluating data that compare the activity of tRNAs which differ in many residues is that the relative contributions of the many tRNA–enzyme contacts to the overall reaction rate are unknown. Even when a tRNA that differs in many nucleotides aminoacylates normally, it is probably not safe to conclude that all the differing positions are unimportant. It is possible that certain nucleotide substitutions could improve K_m or V_{max}, thereby opening the possibility of compensatory effects.

Although the above considerations argue strongly for studying the effect of single nucleotide substitutions on the aminoacylation rate, the interpretations of these data can be complicated as well. A reduction in the reaction rate as a result of changing a nucleotide can be due to either the loss of a helpful contact or the introduction of a harmful one. We have distinguished between these two possiblities for the substitution-sensitive adenosine at position 35 in $tRNA^{Phe}$ by showing that the introduction of an A at that position in $tRNA^{Tyr}$ increases the misacylation rate. In principle, other contacts in the $tRNA^{Phe}$–synthetase interaction could be analyzed in a similar manner. It also should be possible to make several changes in yeast $tRNA^{Tyr}$ such that it aminoacylates just as well as the homologous $tRNA^{Phe}$. It will be interesting to see whether there are different ways to accomplish this result.

Registry No. ApApC, 2760-27-2; phenylalanyl-tRNA synthetase, 9055-66-7; tyrosyl-tRNA synthetase, 9023-45-4.

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Phosphorylation and Methylation of *Physarum* Histone H1 during Mitotic Cycle†

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ABSTRACT: We have shown that the heterogeneity of the typical histone H1 from the lower eukaryote *Physarum polycephalum* seen in sodium dodecyl sulfate ($NaDodSO_4$) gel electrophoresis is due principally to conformational effects produced by postsynthetic modifications rather than primary sequence variants. We have also shown by labeling with L-[*methyl*-^{3}H]methionine and amino acid analysis that *Physarum* H1 in addition to already known phosphorylation undergoes methylation on ϵ-NH_2 groups of several of its lysines. The analysis of *Physarum* H1 phosphorylation by high-resolution acetic acid–urea gel electrophoresis revealed that it could accept up to more than 20 phosphates per molecule. The mitotic cycle analysis of postsynthetic modifications of *Physarum* H1 showed that (a) H1 undergoes superphosphorylation in mitosis accepting from about 14 to more than 20 phosphates per molecule and (b) it is not totally dephosphorylated in the following cycle retaining from about 8 to about 16 phosphates per molecule in G2 phase. The results of this work are consistent with the interpretation that the newly synthesized H1 is deposited on DNA in nonmethylated and nonphosphorylated forms and its methylation (which is irreversible) precedes its phosphorylation in the course of chromatin maturation. The studies of *Physarum* H1 bisected with chymotrypsin indicate that most of the phosphorylation sites including the superphosphorylation in mitosis are localized in a larger COOH-terminal part of the molecule. The analysis of highly phosphorylated *Physarum* H1 by high-resolution $NaDodSO_4$ gel electrophoresis showed the existence of several discrete conformational subspecies resembling in their electrophoretic appearance the true sequence variants of mammalian H1.

Histone H1 plays a key role in the structural transitions of the basic 100-Å nucleosomal filament into the thick chromatin fiber, the 300-Å solenoid (Thoma et al., 1979). On the other hand, H1 shows a considerable degree of variability. Both the sequence variants of H1 and the postsynthetically modified H1 molecules usually occur at the same time in the same chromatin (Hohmann, 1983; Cole, 1984). This together has led to the suggestion that H1 through its different sequence variants and chemically modified subspecies (mostly by phosphorylation) may specifically influence the local conformation of chromatin and through it the genetic activity

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