Biochemical and physical characterization of an unmodified yeast phenylalanine transfer RNA transcribed in vitro

(T7 RNA polymerase/modified nucleotides/amination/melting profile/tRNA tertiary structure)

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ABSTRACT A recombinant plasmid was constructed with six synthetic DNA oligomers such that the DNA sequence corresponding to yeast tRNA\textsuperscript{Phe} is flanked by a T7 promoter and a BstNI restriction site. Runoff transcription of the BstNI-digested plasmid with T7 RNA polymerase gives an unmodified tRNA of the expected sequence having correct 5' and 3' termini. This tRNA\textsuperscript{Phe} transcript can be specifically aminoaoylated by yeast phenylalanyl-tRNA synthetase and has a \(K_m\) only 4-fold higher than that of the native yeast tRNA\textsuperscript{Phe}. The \(V_{max}\) is independent of Mg\textsuperscript{2+} concentration, whereas the \(V_{max}/K_m\) is very dependent on Mg\textsuperscript{2+} concentration. Comparison of the melting profiles of the native and the unmodified tRNA\textsuperscript{Phe} at different Mg\textsuperscript{2+} concentrations suggests that the unmodified tRNA\textsuperscript{Phe} has a less stable tertiary structure. Using one additional DNA oligomer, a mutant plasmid was constructed having a guanosine to thymidine change at position 20 in the tRNA gene. A decrease in \(V_{max}/K_m\) by a factor of 14 for aminoaoylation of the mutant tRNA\textsuperscript{Phe} transcript is observed.

A useful approach for understanding the relationship between structure and function of tRNA involves the physical and biochemical analysis of variant tRNA molecules. A number of methods have been used to create mutant tRNAs in which one or more nucleotides have been altered. Various mutagenesis techniques have produced a large number of mutant tRNA genes (1, 2), but often the expression of the mutant gene is blocked at transcription or processing steps such that biochemical amounts of the tRNA cannot be isolated. tRNAs having specific nucleotide substitutions in the anticodon loop and the T9CG loop have been prepared by the removal of these nucleotides with RNase followed by the insertion of an altered oligoribonucleotide with RNA ligase (3, 4). While this approach has produced many valuable data, specific substitution is limited to those regions of the molecule that are susceptible to partial nuclease digestion. Complete synthesis of two tRNAs has been achieved by joining synthetic oligoribonucleotides with RNA ligase (5, 6). Although this would in principle allow for the synthesis of any desired tRNA sequence, the multiple ligation and subsequent purification of intermediates result in a low yield of final product.

It has been shown that the 3' terminus of brome mosaic virus RNA3, synthesized by \textit{in vitro} runoff transcription of cloned DNA, can be specifically aminoaoylated by tyrosyl-tRNA synthetase (7). It therefore seemed clear that a tRNA lacking the modified nucleotides could be synthesized in a similar manner. In this paper, we describe a detailed method for the synthesis of an unmodified yeast tRNA\textsuperscript{Phe} by runoff transcription using T7 RNA polymerase. The aminoaoylation properties of the wild-type tRNA\textsuperscript{Phe} transcript and a mutant transcript will be discussed. A preliminary account of some of this work has been reported earlier (8).

MATERIALS AND METHODS

Plasmid pSP65 was obtained from Promega Biotec (Madison, WI). The oligodeoxyribonucleotides were synthesized on an Applied Biosystems (Foster City, CA) 380B DNA synthesizer. \(\alpha^32\text{P}\text{JCTP (800 Ci/mmol; 1 Ci = 37 GBq)}\), \(\text{H}^3\text{phenylalanine, and }\text{H}^3\text{tyrosine (50-60 Ci/mmol) were purchased from Amersham. }\gamma^32\text{PJGTP (7000 Ci/mmol)}\) was prepared from \(\gamma^32\text{P} (9). T7 RNA polymerase was isolated from \textit{Escherichia coli} BL21 harboring the plasmid pAR1219 and purified to a specific activity of 450,000 units/mg (10). All DNA restriction endonucleases, T4 polynucleotide kinase, and T4 DNA ligase were purchased from New England Biolabs. Homogeneous yeast phenylalanyl-tRNA synthetase (500 units/mg) was a generous gift of P. Remy (Strasbourg, France).

Construction of Plasmid DNA. The six synthetic DNA oligomers (see Fig. 1) were purified by electrophoresis on denaturing 20% polyacrylamide gels. Four oligomers were phosphorylated in separate 20-\mu reaction mixtures containing 50 mM Tris-HCl (pH 7.6), 10 mM MgCl\textsubscript{2}, 5 mM dithiothreitol, 300 \(\mu\)M ATP, 1 \(\mu\)M DNA, and 2 units of T4 polynucleotide kinase. After incubation at 37° for 1 hr, the reaction mixtures were combined and the kinase was heat-inactivated at 60° for 10 min. Stoichiometric amounts of the two remaining oligomers were added and annealed by heating this mixture to 90° and slowly cooling to 25°. Oligomers were joined in a 100-\mu reaction mixture containing 50 mM Tris-HCl (pH 7.8), 20 mM MgCl\textsubscript{2}, 5 mM dithiothreitol, 300 \(\mu\)M ATP, 200 nM DNA, and 10 units of T4 DNA ligase. After incubation for 8 hr at 25°, the ligated 105-base-pair product was purified on a native 10% polyacrylamide gel. One picomole of the purified insert and 0.25 pmol of EcoRI/BamHI-digested plasmid pSP65 were incubated with T4 DNA ligase in 25 \(\mu\)l under the conditions described above for 20 hr at 4°. This reaction mixture was then used directly for transformation of \textit{E. coli} JM83. Transformants harboring plasmid DNA were screened for the 105-base-pair insert using a \textit{Pst I/BamHI} restriction analysis of minilysate plasmid DNA. Both strands of the plasmid (p67YF0) were sequenced in the region of the insert with reverse transcriptase using either the oligomer corresponding to the top strand of the T7 promoter or New England Biolabs no. 1201 as the primer (11). A plasmid (p67YF4) having the guanosine to thymidine change at position +20 in the tRNA gene was constructed by substituting a mutant oligonucleotide and transforming with a mismatched 105-base-pair insert. Minilysate plasmid DNA with this insert was then used for retransformation of \textit{E. coli} JM83 prior to sequencing as a precaution against a single transformant harboring plasmid with both mutant and wild-type insert. The mutant insert was easily found by sequencing the plasmid DNA from several transformants.

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In Vitro Transcription. Template DNA was prepared by BstNI digestion of the plasmid in 0.5 ml containing 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10 mM MgCl₂, 100 μg of plasmid DNA, and 150 units of BstNI. After incubation at 60°C for 1 hr, the reaction mixture was phenol-extracted and the DNA was ethanol-precipitated. A typical 0.5-ml transcription reaction mixture contained 40 mM Tris-HCl (pH 8.1), 6 mM MgCl₂, 1 mM spermidine, 5 mM dithiothreitol, 50 μg of bovine serum albumin per ml, 1.0 mM each NTP, 50 μg of BstNI-digested DNA, 2 units of inorganic pyrophosphatase (Sigma), and 12,000 units of T7 RNA polymerase. After incubation for 1 hr at 42°C, EDTA was added to a final concentration of 50 mM, and the reaction mixture was extracted once with phenol/CHCl₃/isoamyl alcohol (25:24:1), once with CHCl₃/isoamyl alcohol (24:1), and ethanol-precipitated. The transcript was purified to single nucleotide resolution (see Results) by electrophoresis on denaturing 20% polyacrylamide gels (42 × 38 × 0.14 cm) and eluted from the gel in 50 mM KOAc/20 mM EDTA/200 mM KCl for 48 hr at 4°C. After precipitating the RNA with ethanol, residual polyacrylamide and urea were removed by binding the RNA to a 0.2-ml TSK Fractogel-DEAE column, washing with 0.5 ml of 50 mM NaOAc/1 mM EDTA/50 mM NaCl, pH 7.0, and eluting the RNA with 800 mM NaCl in the same buffer. The purified transcript was ethanol-precipitated, washed with 70% ethanol, dried, and stored at a concentration of 20 μg or greater in 5 mM Hepes-KOH/1 mM EDTA, pH 7.5. The 0.5-ml reaction mixtures yielded an average of 0.2 mg of transcript, which corresponds to 300 mol of RNA per mol of DNA template. Yeast tRNA⁸¹phe purchased from Boehringer Mannheim was purified in the same manner.

Aminoacylation of tRNAs. Kinetic reactions were carried out in 60 μl of either buffer A: 30 mM Hepes KOH, pHe 7.45/10 μM [³H]phenylalanine/2 mM ATP/15 mM MgCl₂/-25 mM KCl/4 mM dithiothreitol, or buffer B: 25 mM Tris HCl, pH 8.0/5 μM [³H]phenylalanine/1 mM ATP/2.0 mM MgCl₂/1.0 mM spermine/100 μM dithiothreitol. tRNA samples were heated to 60°C and slowly cooled to 25°C prior to addition to the aminoacylation reaction mixture. tRNA concentrations ranged from 0.03 μM to 2.6 μM and the reactions were initiated by the addition of synthetase to final concentrations ranging between 0.07 and 0.3 unit/ml. Incubation was at 37°C. Seven-microliter aliquots were removed at 10-sec intervals, spotted on Whatman 3MM paper, and treated as described (3). Kₐₐ and Vₐₐₐₐₐ values were obtained from an Eadie-Hofstee analysis of the initial rates by using five different concentrations of tRNA at the fixed synthetase concentration. Misacylation of the tRNAs with purified yeast tyrosyl-tRNA synthetase was carried out as described (12).

Thermal Melting Profiles. Thermal denaturation profiles of the tRNAs were obtained using a Gilford 2400 spectrophotometer interfaced to a microcomputer. The temperature was increased from 25°C to 85°C at a rate of 1.0°C/min. The tRNA samples were heated to 85°C and slowly cooled to 25°C prior to obtaining the thermal denaturation profiles.

RESULTS

In Vitro Transcription and Characterization of an Unmodified tRNA. Six synthetic DNA oligomers were used to construct the plasmid p67YF0, which contains a T7 promoter directly adjacent to the yeast tRNA⁸¹phe gene and a BstNI restriction site at the 3′ end of the gene (Fig. 1). Digestion of p67YF0 by BstNI results in a linear DNA template having a single 5′ overhanging thymidine that is complementary to the 3′-terminal adenosine of the tRNA. Runoff transcription of the BstNI-digested p67YF0 would be expected to give a 76-nucleotide RNA with a sequence identical to an unmodified yeast tRNA⁸¹phe. Analysis of the transcription reaction on a 20% polyacrylamide gel reveals a single RNA product that comigrates with yeast tRNA⁸¹phe (Fig. 2A). To confirm that transcription initiated at guanosine +1 in the tRNA gene, [γ-³²P]GTP-labeled RNA was subjected to partial T1 digestion and analyzed by gel electrophoresis. As shown in Fig. 2B, the guanosine residues appeared at the expected positions in the RNA sequence, which indicates that transcription initiated exclusively at position +1. Partial digestion of this 5′-end-labeled RNA with other specific ribonucleases confirmed that the transcript was an accurate RNA copy of the known DNA sequence (data not shown). The 3′-terminal nucleotide was determined by 3′-labeling the transcript with [⁵'-³²P]PpCp (13) followed by total RNase T2 digestion and separation of the products by two-dimensional TLC (14). As shown in Fig. 2C, ~90% of the transcription product possessed the expected 3′-terminal adenosine, while the remaining 10% terminated with either guanosine or cytidine residues. These
minor products resulted from the incorporation of one or two additional non-template-directed nucleotides on the 3' terminus (15) and could be removed by preparative high-resolution polyacrylamide gel electrophoresis. All subsequent experiments used this highly purified material (Fig. 3).

To replace the 5'-terminal triphosphate present on the transcript with a 5' monophosphate, GMP was used to prime the transcription reaction. As shown in Fig. 4, increasing concentrations of GMP in the transcription reaction mixture reduced the incorporation of [γ-32P]GTP label at the 5' terminus of the full-length transcript without altering the amount of [α-32P]CTP incorporated. The presence of a 5'-terminal monophosphate for the GMP-primed reaction was confirmed by complete RNase T2 digestion of the [α-32P]CTP-labeled transcript followed by two-dimensional TLC analysis (Fig. 4 Inset).

**Aminoacylation of the tRNA\(^{\text{Phe}}\) Transcripts.** The tRNA\(^{\text{Phe}}\) transcripts having either a 5' monophosphate or triphosphate were compared to yeast tRNA\(^{\text{Phe}}\) for phenylalanine acceptor activity using yeast phenylalanyl-tRNA synthetase. As shown in Fig. 5, both tRNA transcripts could be aminoacylated by phenylalanyl-tRNA synthetase to >1300 pmol of phenylalanine/A\(_{260}\) unit of tRNA. This corresponds to >90% aminoacylation and demonstrates that the transcripts are essentially pure and competent for aminoacylation.

The kinetics of aminoacylation were determined for the three tRNAs in buffer A, which maximizes the activity of phenylalanyl-tRNA synthetase (Table 1). The \(K_m\) and \(V_{max}\) values obtained for the yeast tRNA\(^{\text{Phe}}\) in this buffer are similar to those previously reported (3). Both tRNA\(^{\text{Phe}}\) transcripts have a \(K_m\) 4 times higher than tRNA\(^{\text{Phe}}\) and a slightly lower \(V_{max}\), resulting in a 5 times lower \(V_{max}/K_m\). This clearly demonstrates that the absence of the 14 modifications normally found in yeast tRNA\(^{\text{Phe}}\) has only a modest effect on the aminoacylation kinetics under these conditions. Furthermore, the number of phosphates on the 5'-terminal guanosine has no effect on the reaction kinetics.

Loftfield et al. (16) have shown that the aminoacylation buffer conditions can greatly affect the rate of misacylation of noncognate tRNAs by yeast phenylalanyl-tRNA synthetase. Therefore, the aminoacylation kinetics of yeast tRNA\(^{\text{Phe}}\) and the tRNA\(^{\text{Phe}}\) transcript having a 5'-terminal triphosphate were compared in buffer B, which maximizes the accuracy of phenylalanyl-tRNA synthetase. As shown in Table 1, the respective \(K_m\) values for tRNA\(^{\text{Phe}}\) and the transcript are the same in both buffers, whereas the \(V_{max}\) of the transcript is comparatively much lower in buffer B. This results in a decrease by a factor of 17 in \(V_{max}/K_m\) for the transcript in this buffer. Thus, the absence of the modified nucleotides has a much larger effect on the aminoacylation kinetics in the high-accuracy buffer.

Since the major difference between these two buffers is the presence of spermine and the much lower Mg\(^{2+}\) concentration in buffer B, the Mg\(^{2+}\) dependence on the velocity of aminoacylation was investigated. Because the \(K_m\) was unaffected by the Mg\(^{2+}\) concentration, the initial velocities (\(V_0\)) of aminoacylation for yeast tRNA\(^{\text{Phe}}\) and the tRNA\(^{\text{Phe}}\) transcript were determined at their respective \(K_m\) values at different Mg\(^{2+}\) concentrations. As shown in Fig. 6, the two tRNAs show a very different Mg\(^{2+}\) dependence on the rate of aminoacylation. At low Mg\(^{2+}\) concentrations, yeast tRNA\(^{\text{Phe}}\) is a much better substrate than the transcript, whereas above 8 mM Mg\(^{2+}\), the \(V_0\) values for both tRNAs are essentially the same. Loftfield et al. (16) reported that the \(V_{max}\) for misacylation of the noncognate E. coli tRNA\(^{\text{Val}}\) by phenylalanyl-tRNA synthetase is reduced by as much as a factor of 1000 in the presence of the lowered Mg\(^{2+}\) concentration. Although

![Fig. 3](image-url)  
Fig. 3. The tRNA\(^{\text{Phe}}\) transcript folded in a cloverleaf secondary structure. The mutant tRNA transcript (guanosine-20→uridine-20) is indicated.
Table 1. Aminoacylation kinetics of tRNA transcripts

<table>
<thead>
<tr>
<th>tRNA</th>
<th>$K_m$ (nM)</th>
<th>$V_{max}$ (pmol·min$^{-1}$·mg$^{-1}$)</th>
<th>$V_{max}/K_m$ (relative)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yeast tRNA$^{Phe}$</td>
<td>96</td>
<td>700</td>
<td>1.0</td>
</tr>
<tr>
<td>YF0 (pppG)</td>
<td>380</td>
<td>580</td>
<td>0.21</td>
</tr>
<tr>
<td>YF0 (pG)</td>
<td>380</td>
<td>570</td>
<td>0.21</td>
</tr>
<tr>
<td>YF4 (pppG)</td>
<td>2100</td>
<td>230</td>
<td>0.015</td>
</tr>
<tr>
<td>YF4 (pG)</td>
<td>2200</td>
<td>230</td>
<td>0.015</td>
</tr>
<tr>
<td>Buffer B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yeast tRNA$^{Phe}$</td>
<td>100</td>
<td>360</td>
<td>1.0</td>
</tr>
<tr>
<td>YF0 (pppG)</td>
<td>380</td>
<td>79</td>
<td>0.058</td>
</tr>
<tr>
<td>YF4 (pppG)</td>
<td>2100</td>
<td>32</td>
<td>0.0042</td>
</tr>
</tbody>
</table>

YF0 corresponds to the tRNA$^{Phe}$ transcribed from p67YF0 and YF4 corresponds to the mutant p67YF4. pppG indicates transcript having a 5’ triphosphate, and pG indicates a 5’ monophosphate.

The effect of Mg$^{2+}$ on the $V_{max}$ for the transcript is not nearly as great, the aminoacylation of the transcript by phenylalanyl-tRNA synthetase somewhat resembles that of a noncognate tRNA-synthetase interaction.

To test the possibility that a tRNA$^{Phe}$ lacking the modified nucleotides could be recognized more easily by a noncognate synthetase, the rate of misacylation of yeast tRNA$^{Phe}$ and the tRNA$^{Phe}$ transcript by yeast tyrosyl-tRNA synthetase were compared. It was found that the rates of misacylation for both yeast tRNA$^{Phe}$ and the transcript were at least 3000 times lower than that of the control yeast tRNA$^{Tyr}$. While this result was not unexpected for yeast tRNA$^{Phe}$, the inability of tyrosyl-tRNA synthetase to misacylate the tRNA$^{Phe}$ transcript suggests that the modified nucleotides are not required to suppress misacylation by at least one noncognate synthetase.

**Thermal Stability of the tRNA Transcript.** The thermal melting profiles of yeast tRNA$^{Phe}$ and the tRNA$^{Phe}$ transcript were determined as a function of Mg$^{2+}$ concentration. In the absence of Mg$^{2+}$, both tRNAs show an initial broad transition at $\approx 60^\circ$C followed by a sharper transition at $\approx 70^\circ$C (Fig. 7). As the concentration of Mg$^{2+}$ is increased, the transition for the transcript slowly becomes sharper and the melting temperature increases until $\approx 8$ mM Mg$^{2+}$, at which point point hydrolysis of the tRNA prevents further reliable data collection. In contrast, yeast tRNA$^{Phe}$ shows a single transition at 1.0 mM Mg$^{2+}$ that is 6°C higher than the transcript. This suggests that the transcript has a less-stable structure than the native yeast tRNA$^{Phe}$. This is consistent with the finding that the absence of the ribosylthymine modifications destabilizes E. coli tRNA$^{Met}$ (18).

**Aminoacylation of a Mutant tRNA$^{Phe}$ Transcript.** Because the aminoacylation kinetics are essentially the same for both the unmodified and the native tRNA$^{Phe}$ in the presence of buffer A, a kinetic analysis of mutant tRNA$^{Phe}$ transcripts should allow for a detailed evaluation of the phenylalanyl-tRNA synthetase recognition site. A mutant tRNA in which guanosine-20 was changed to uridine was prepared by runoff transcription of BstNI-digested p67YF4 (Fig. 3). This particular mutation was chosen for several reasons. First, guanosine-20 does not participate in any secondary or tertiary interactions (19). Therefore, any observed effect on aminoacylation as a result of this change would not be expected to be the consequence of an altered tertiary structure. Second, tRNA$^{Phe}$ is the only known yeast tRNA with a guanosine at position 20, suggesting that it may be a discriminatory nucleotide (20). Finally, kethoxalation of guanosine-20 reduces the extent of aminoacylation (21). As shown in Fig. 5, the mutant tRNA transcripts with either the 5’ monophosphate or triphosphates could be fully aminoacylated. However, the kinetics of aminoacylation in buffers A and B revealed that both mutant tRNA transcripts exhibited a 5 times higher $K_m$ and a 2 times lower $V_{max}$ than the wild-type tRNA$^{Phe}$ transcript (Table 1). Therefore, the single nucleotide substitution at position +20 has a large effect on aminoacylation, and the discrimination between the mutant and the wild-type tRNA$^{Phe}$ transcript remains the same in both buffers.

**DISCUSSION**

The method we have described for the in vitro synthesis of an unmodified tRNA by runoff transcription could be used for synthesizing virtually any tRNA, provided that the 5’-terminal sequence corresponds to an active T7 promoter. The fact that we observe efficient transcription with a mutant GCGGA promoter and the report of a number of other active promoters having changes in the $+1$ to $+5$ region (15) indicate that this restriction may not be too great. If an active DNA template can be prepared, this is an excellent method for synthesizing very large amounts of tRNA for both biochemical and biophysical studies. Recently, in a 10-ml transcription reaction mixture that contained 26 mM MgCl$_2$ and 5 mM each NTP, 15 mg of tRNA was obtained.

The availability of a completely unmodified tRNA makes it possible to evaluate the role of the modifications in the structure and function of the molecule. The ability of the tRNA transcript to aminoacylate with virtually normal kinetics in the high Mg$^{2+}$ buffer strongly suggests that a direct association between phenylalanyl-tRNA synthetase and any
of the modifications is unlikely. This conclusion is consistent with most of the available data on the role of the modified nucleotides on tRNA-synthetase interactions. For example, undermodified E. coli tRNA\(^{Phe}\) and tRNA\(^{Trp}\) are fully active in aminoacylation (22). The absence of the modifications on nucleotides 34 and 37 in the anticodon loop of yeast tRNA\(^{Phe}\) has little effect on the aminoacylation rate (3). Perhaps the strongest evidence for the direct role of a modification in the aminoacylation reaction comes from the data of Roe et al. (23). They showed that \textit{in vitro} methylation of E. coli tRNA\(^{Phe}\) at guanosine-10, which is normally found in yeast tRNA\(^{Phe}\), results in a 10-fold increase in \(V_{\text{max}}\) of aminoacylation by yeast phenylalanyl-tRNA synthetase. However, \textit{Schizosaccharomyces pombe} tRNA\(^{Phe}\) lacks this modification but aminoacylation by yeast phenylalanyl-tRNA synthetase has virtually the same kinetics as that of yeast tRNA\(^{Phe}\) (24). Thus, the interpretation of these results, which are based on heterologous systems, is unclear.

The ability of the tRNA\(^{Phe}\) transcript to aminoacylate normally in the presence of 15 mM Mg\(^{2+}\) also suggests that the absence of the modified nucleotides do not greatly alter the tertiary folding of the tRNA. This conclusion is consistent with the crystal structure of yeast tRNA\(^{Phe}\), which reveals that none of the modifications is directly involved in any secondary or tertiary hydrogen-bonding interactions (25). All available experimental data on the structure of unmodified tRNAs also indicate that the molecule is folded normally. Analysis of the tertiary structure of the partially modified yeast tRNA\(^{Phe}\) precursor using chemical and enzymatic probes revealed a structure very similar to the mature tRNA\(^{Phe}\) (26). The rate of Pb\(^{2+}\)-induced cleavage at nucleotide 17 for the unmodified tRNA transcript is only 2 times slower than that of the native tRNA\(^{Phe}\) (27). Finally, the NMR spectrum of the tRNA transcript in H\(_2\)O indicates that most of the tertiary interactions that can be detected by NMR are present (K. Hall, personal communication).

Unlike the results obtained at 15 mM Mg\(^{2+}\), below 5 mM Mg\(^{2+}\) the rate of aminoacylation for the unmodified tRNA\(^{Phe}\) transcript is much slower than that of the native yeast tRNA\(^{Phe}\). This correlates with the fact that at lower Mg\(^{2+}\) concentrations the unmodified tRNA transcript shows a much broader melting profile and suggests that the unmodified tRNA transcript may not have the same structure as the native tRNA\(^{Phe}\) under these conditions. It is interesting to note that the tight-binding Mg\(^{2+}\) ion in the anticodon loop of the yeast yeast tRNA\(^{Phe}\) is coordinated through water to both Y37 and Y39 (28). In addition, the Mg\(^{2+}\) ion, which serves to stabilize the sharp bend in the polynucleotide chain at nucleotide 10, is also in close proximity to mG-10 normally found in native yeast tRNA\(^{Phe}\). Therefore, the absence of these modifications could result in altered binding of the Mg\(^{2+}\) ions.

The ease of synthesizing mutant tRNAs by this method allows for a number of structure-function experiments. Two such experiments were carried out in this work. In the first, it was shown that introducing a 5′-terminal triphosphate on the tRNA had no effect on the kinetics of aminoacylation. Sprinzl and Graeser (29) showed that the aminoacylation of yeast tRNA\(^{Phe}\) by phenylalanyl-tRNA synthetase is not affected by removal of the 5′ phosphate. The fact that the 5′ phosphate lies along the top of the aminocyl stem in the L-shaped structure suggests that this part of the tRNA is either not in contact with the synthetase or the space and charge occupied by the two additional phosphates can be accommodated by the enzyme. Second, it was shown that the overall rate of aminoacylation for the tRNA transcript with the guanosine to uridine change at position 20 is 14 times lower than that of the wild-type transcript. Based on the crystal structure, we do not expect this modified tRNA to have a different tertiary structure. We have shown elsewhere (27) that the Pb\(^{2+}\)-induced cleavage properties of the uridine-20 transcript are the same as that of the wild-type transcript. Preliminary NMR analysis of the mutant tRNA also indicates that it is not structurally different from the wild-type transcript (K. Hall, personal communication). It is tempting to conclude that guanosine-20 interacts directly with yeast phenylalanyl-tRNA synthetase. However, it is important to note that in yeast tRNA\(^{Phe}\), the N\(_7\) and O\(_6\) of guanosine-20 are coordinated (presumably through water) to one of the tightly bound Mg\(^{2+}\) ions (30). It is therefore also possible that the loss of these functional groups in the mutant tRNA results in a lower affinity for a Mg\(^{2+}\) ion, which also interacts directly with the synthetase.

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