The Role of 5-Methylcytidine in the Anticodon Arm of Yeast tRNA^{Phe}: Site-Specific Mg^{2+} Binding and Coupled Conformational Transition in DNA Analogs†

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ABSTRACT: The tDNA$^{Phe}_{AC}$, d(CCAGACTGAAGAU$_{13}$m^{5}C$_{14}$U$_{15}$GG), with a DNA sequence similar to that of the anticodon stem and loop of yeast tRNA^{Phe}, forms a stem and loop structure and has an Mg^{2+}-induced structural transition that was not exhibited by an unmodified tDNA$^{Phe}_{AC}$ d(T$_{13}$C$_{14}$T$_{15}$) [Guenther, R. H., Hardin, C. C., Sierzputowska-Gracz, H., Dao, V., & Agris, P. F. (1992) *Biochemistry* (preceding paper in this issue)]. Three tDNA$^{Phe}_{AC}$ molecules having m^{5}C$_{14}$, tDNA$^{Phe}_{AC}$ d(U$_{13}$m^{5}C$_{14}$U$_{15}$), d(U$_{13}$m^{5}C$_{14}$T$_{15}$), and d(T$_{13}$m^{5}C$_{14}$U$_{15}$), also exhibited Mg^{2+}-induced structural transitions and biphasic thermal transitions ($T_m \approx 23.5$ and 52 °C), as monitored by CD and UV spectroscopy. Three other tDNA$^{Phe}_{AC}$, d(T$_{13}$C$_{14}$T$_{15}$), d(U$_{13}$C$_{14}$U$_{15}$), and d(A$_7$;U$_{13}$m^{5}C$_{14}$U$_{15}$) in which T$_7$ was replaced with an A, thereby negating the T$_7$·A$_{10}$ base pair across the anticodon loop, had no Mg^{2+}-induced structural transitions and only monophasic thermal transitions (T_m of ≈52 °C). The tDNA$^{Phe}_{AC}$ d(U$_{13}$m^{5}C$_{14}$U$_{15}$) had a single, strong Mg^{2+} binding site with a K_d of 1.09×10^{-6} M and a ΔG of −7.75 kcal/mol associated with the Mg^{2+}-induced structural transition. In thermal denaturation of tDNA$^{Phe}_{AC}$ d(U$_{13}$m^{5}C$_{14}$U$_{15}$), the ^{1}H NMR signal assigned to the imino proton of the A$_5$·dU$_{13}$ base pair at the bottom of the anticodon stem could no longer be detected at a temperature corresponding to that of the loss of the Mg^{2+}-induced conformation from the CD spectrum. Therefore, we place the magnesium in the upper part of the tDNA hairpin loop near the A$_5$·dU$_{13}$ base pair, a location similar to that in the X-ray crystal structure of native, yeast tRNA^{Phe}. We conclude that m^{5}C in the anticodon stems of the tDNA analog, and of tRNA, facilitates a site-specific Mg^{2+} binding without 2′-OHs and a conformational change in the anticodon loop, which for tRNA has important functional implications.

A fundamental understanding of the stability and dynamics of nucleic acid stems and loops, hairpin configurations, is essential for an understanding of their biological functions and ligand-binding interactions. Short DNA and RNA hairpin structures are used as models to study the mechanism(s) of folding of nucleic acid molecules as it occurs in the cell. Investigations of these structures also provide insight into nucleic acid dynamics. Studies of selected base sequences of both DNA and RNA under physiological conditions have shown that they are stable as hairpins rather than as duplexes (Guenther et al., 1992; Blommers et al., 1991; Germann et al., 1990; Williamson & Boxer, 1989; Puglisi et al., 1990; Varani et al., 1991; Heus & Pardi, 1991). These studies have also demonstrated that hairpins form energetically favorable structures that are in the traditional sense without loops, but consist of Watson–Crick base-paired stems, noncanonical nucleoside pairings across what normally would be considered a loop, and turns comprised of as little as two nucleosides.

The folding of DNA hairpins in solution has been shown to be dependent on the sequence of the loop and stem (Blommers et al., 1991; Orbons et al., 1987; Williamson & Boxer, 1989; Blommers et al., 1989; Germann et al., 1990). DNA hairpins with four-membered loops containing complementary bases at loop positions 1 and 4 can form a base pair across the loop and a two-base turn (Blommers et al., 1991, 1989). In a study of three sequence-related DNA hairpins, the 5-membered loops adopted both a 5′ stack conformation (three bases stacked with the 5′ side of the stem) and a 3′ stack (three bases stacked with the 3′ side of the stem) as a consequence of the sequence-dependent location of the turn in the loop (Williamson & Boxer, 1989). Recently, we found that the DNA analog of a tRNA hairpin formed two additional hydrogen bonds across a seven-membered loop and that the position of the two-base turn created a 3′ stack (Guenther et al., 1992). When duplex base stacking of a DNA stem is taken into consideration, the differences between the A and B forms are not as clear; the B form has greater variability of backbone conformation (C2′-endo vs C3′-endo), and more parameters are required to define structure, particularly local structure (Privé et al., 1991; Travers, 1992). Stable conformations of certain DNA sequences, DNA hairpins in particular, may be similar to the sequence-dependent stability that has been observed in RNA hairpins (Puglisi et al., 1990; Varani et al., 1991; Heus & Pardi, 1991).

There are only three fundamental chemical differences between RNA and DNA: the 2′-OH of the ribose instead of the 2′ hydrogen; the uracil base instead of thymine; and the increased number and variety of modified nucleosides in RNA, especially tRNA (Gehrke et al., 1990). Until recently, it has not been practical to investigate in detail the individual contributions of the 2′-OH, U vs T, or that of specific modified nucleosides to nucleic acid conformation and stability. Methyl groups in DNA had been shown to alter DNA conformation, as well as the sequence-dependent recognition by DNA-binding proteins (Hager, 1990). Depending upon sequence position pyrimidine 5-methyl groups significantly affect the degree of curvature of a DNA molecule. Methylation of dC in DNA to d(m^{5}C) increases the melting temperature of the DNA.

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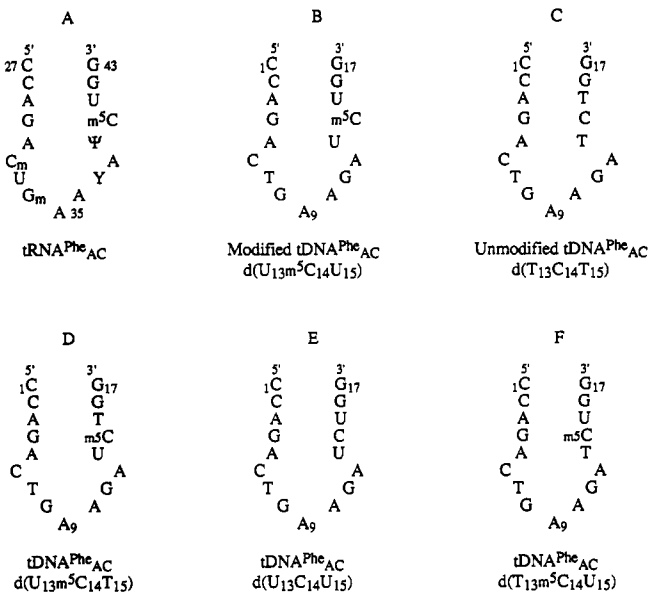

FIGURE 1: Sequence comparisons and secondary structures of the yeast tRNA^{Phe} anticodon stem/loop and the five different tDNA$^{Phe}_{AC}$ analogs. (A) Stem/loop secondary structure formed in the anticodon arm of native yeast tRNA^{Phe}. Modified nucleotides include the 2'-*O*-methyl derivatives of C (Cm) and G (Gm), 5-methylcytidine (m^{5}C), pseudouridine (Ψ), and the hypermodified guanosine derivative wye (Y). The molecules used in this study are DNA analogs of the yeast tRNA^{Phe} anticodon stem and loop, and they are shown in panels B–F. (B) Modified tDNA$^{Phe}_{AC}$ containing the RNA-like sequence d(U$_{13}$m^{5}C$_{14}$U$_{15}$) on the stem. (C) Unmodified tDNA$^{Phe}_{AC}$ with the sequence d(T$_{13}$C$_{14}$T$_{15}$) on the stem. (D) A tDNA$^{Phe}_{AC}$ with d(U$_{13}$m^{5}C$_{14}$T$_{15}$). (E) A tDNA$^{Phe}_{AC}$ with the sequence d(U$_{13}$C$_{14}$U$_{15}$). (F) A tDNA$^{Phe}_{AC}$ with the sequence d(T$_{13}$m^{5}C$_{14}$U$_{15}$) on the stem.

The major physicochemical change due to methylation is thought to be an increase in the molecular polarizability of the pyrimidine, leading to increased base stacking energies (Sowers et al., 1987). The methyl groups of tRNA have been shown to have location-dependent motional characteristics, with those in the anticodon being more motionally restricted (Schmidt et al., 1987). Studies of tRNA transcribed in vitro, and thus devoid of modified nucleosides, suggest that modified nucleosides may facilitate the binding of Mg^{2+} and induce subsequent conformational changes that are important for quantitative and correct aminoacylation of the molecule (Hall et al., 1989; Sampson & Uhlenbeck, 1988; Sampson et al., 1992; Perret et al., 1990).

In order to determine the contributions of the ribose 2'-OH and modified nucleosides to conformation, stability, and ion binding of a biologically relevant RNA hairpin structure, we synthesized and studied two DNA heptadecamer analogs to the anticodon stem and loop of yeast tRNA^{Phe} (Guenther et al., 1992). Results of CD and NMR studies showed that addition of Mg^{2+} induced a structural transition in a tDNA hairpin with a base sequence which was modified to more closely resemble the RNA, tDNA$^{Phe}_{AC}$[1] d(U$_{13}$m^{5}C$_{14}$U$_{15}$) (Figure 1). In contrast, the conformation of the unmodified tDNA$^{Phe}_{AC}$ d(T$_{13}$C$_{14}$T$_{15}$) was not affected by addition of Mg^{2+}. The Mg^{2+}-induced structural transition in tDNA$_{AC}$ resulted in a hairpin with two additional hydrogen bonds across the loop, between C_6 and G_{11}, and T_7 and A_{10} (Figure 1B), a two-base turn rather than the more commonly depicted seven-membered loop, and a single-base bulge. Site-specific Mg^{2+} binding and a subsequent conformational change have been observed for the anticodon loop of yeast tRNA^{Phe} (Striker et al., 1989). Here we report that m^{5}C$_{14}$ is required for binding of one Mg^{2+} per tDNA$^{Phe}_{AC}$ and that binding of the divalent cation results in a conformational transition which significantly stabilizes the structure.

[1] Abbreviations: CD, circular dichroism; tDNA$^{Phe}_{AC}$, the DNA analog corresponding to the anticodon stem–loop of phenylalanyl transfer RNA.

MATERIALS AND METHODS

DNA Synthesis, Purification, and Sample Preparation. Six single-stranded DNA oligonucleotides, each 17 residues in length, were synthesized using standard phosphoramidite chemistry (Sinha et al., 1984). The DNA nucleoside sequences correspond to those of the anticodon stem and loop of yeast tRNA^{Phe} with several modified bases. The unmodified DNA sequence, d(C$_1$CAGACTGAAGAT$_{13}$C$_{14}$T$_{15}$GG$_{17}$), is designated unmodified tDNA$^{Phe}_{AC}$. One of the oligonucleotides was synthesized with three base modifications; the sequence d(T$_{13}$C$_{14}$T$_{15}$) was changed to d(U$_{13}$m^{5}C$_{14}$U$_{15}$), i.e., d(CCAGACTGAAGAU$_{13}$m^{5}C$_{14}$U$_{15}$GG), and the oligomer designated modified tDNA$^{Phe}_{AC}$ d(U$_{13}$m^{5}C$_{14}$U$_{15}$). Three of the tDNA$^{Phe}_{AC}$ sequences that were synthesized have two base changes within the sequence d(T$_{13}$C$_{14}$T$_{15}$) and are designated as follows: tDNA$^{Phe}_{AC}$ d(U$_{15}$m^{5}C$_{14}$T$_{15}$); tDNA$^{Phe}_{AC}$ d(U$_{13}$C$_{14}$U$_{15}$); and tDNA$^{Phe}_{AC}$ d(T$_{13}$m^{5}C$_{14}$U$_{15}$). The RNA sequence of native yeast tRNA$^{Phe}_{AC}$ (Figure 1A), the DNA sequences of modified (panel B) and unmodified (panel C) tDNA$^{Phe}_{AC}$, and those of the three partially modified tDNA$^{Phe}_{AC}$ molecules (panels D–F) are all shown in Figure 1. In addition, a sixth sequence was synthesized in which T_7 was changed to an A, and the sequence d(T$_{13}$C$_{14}$T$_{15}$) was changed to d(U$_{13}$m^{5}C$_{14}$U$_{15}$), i.e., d(CCAGACA$_7$GAAGAU$_{13}$m^{5}C$_{14}$U$_{15}$GG). This oligomer is designated tDNA$^{Phe}_{AC}$ d(A$_7$;U$_{13}$m^{5}C$_{14}$U$_{15}$). The synthesized oligomers were purified as previously reported (Guenther et al., 1992). In order to further diminish any possible metal ion content, the aqueous solutions of the samples were reprecipitated with a solution of 50% acetone and 50% ethanol at –20 °C for 2 h. Counterion (25 mM LiCl) was also added to facilitate precipitation; the precipitates were collected by centrifugation.

The concentrations of the different tDNA$^{Phe}_{AC}$ stock solutions were determined using the absorbance at 260 nm (HP 8451A diode array spectrophotometer) of solutions diluted with 10 mM sodium phosphate buffer at pH 7.0. All of the samples were adjusted to concentrations of 0.8 absorbance unit at 260 nm in 10 mM sodium phosphate buffer at pH 7.0 before the addition of Mg^{2+} in order to ensure that equimolar amounts of DNA oligonucleotides were used. The extinction coefficient of the single-stranded form was 1.60×10^6 M^{-1} cm^{-1} (Cantor & Warshaw, 1970).

Circular Dichroism Spectropolarimetry. Circular dichroism (CD) spectra were collected using a Jasco J600 spectropolarimeter and an interfaced IBM PC/2 microcomputer. To prevent dilution of the sample upon titration with Mg^{2+}, the appropriate amount of 25 mM $MgCl_2$ solution was vacuum dried in tubes and then tDNA$^{Phe}_{AC}$ samples were added to each tube to dissolve the $MgCl_2$. Temperatures were maintained by placing the samples in 0.1-, 1-, or 2-mL cylindrical cells with an external water-circulating jacket. The temperature-dependent reaction rates of the Mg^{2+}-induced structural transition were determined by first equilibrating the samples at 15 °C, and then quickly changing the circulating water to a second water bath at higher temperature (25, 26, 27, 28.5, 30, or 35 °C). Sample temperatures equilibrated within seconds after each sample temperature change. The CD spectra were collected every 0.5 s after this temperature change until the first plateau was reached, indicating a return to equilibrium.

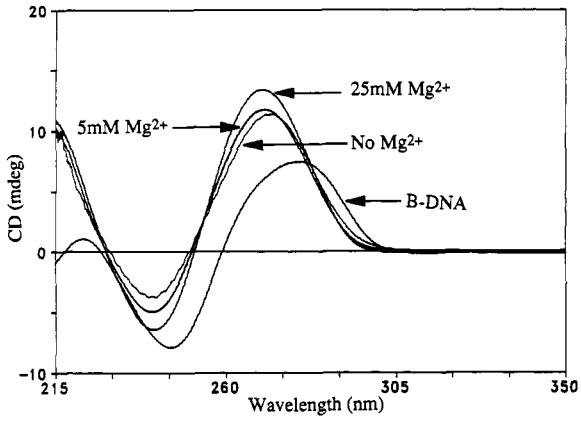

FIGURE 2: Effect of Mg^{2+} on the CD spectrum of modified $tDNA^{Phe}_{AC}$. Circular dichroism spectra of modified $tDNA^{Phe}_{AC}$ in 10 mM sodium phosphate solution (pH 7) were collected at different Mg^{2+} concentrations (0, 5, or 25 mM of $MgCl_2$) at 10 °C. For comparison, the CD spectrum of a B-form DNA (Johnson & Tinoco, 1969) is also shown in the figure. The double-stranded B-form DNA is from bacteriophage λ in 10 mM sodium phosphate (pH 7).

Thermal Denaturation Analysis. The "melting" curves of different oligonucleotides were obtained on a Gilford Response System spectrophotometer. The temperature changes were controlled by Response II Thermal Programming software. The temperature was increased from 15 to 85 °C at a rate of 0.5 °C/min. The reaction was reversible, resulting in renaturation under these conditions since minimal hysteresis was noted upon cooling.

NMR Methods. NMR data were obtained as previously reported (Guenther et al., 1992).

RESULTS

A synthesized DNA oligonucleotide, the modified $tDNA^{Phe}_{AC}$ $d(U_{13}m^5C_{14}U_{15})$ (Figure 1B), with a sequence similar to that of the anticodon stem and loop of yeast $tRNA^{Phe}_{AC}$ (Figure 1A) undergoes a Mg^{2+}-induced structural transition (Guenther et al., 1992). This Mg^{2+}-induced structural change did not occur with the unmodified $tDNA^{Phe}_{AC}$ $d(T_{13}C_{14}T_{15})$ oligomer that had a standard DNA sequence (Figure 1C). The structural transition, as detected by CD spectropolarimetry, resulted in a small but highly reproducible shift of the UV maximum to shorter wavelengths, from 272 to 269 nm, and away from the 280-nm maximum normally found with B-form DNA (Figure 2). Increased Mg^{2+} concentrations, from 5 to 25 mM, resulted in amplification of the peaks at 269 nm (maximum) and 241 nm (minimum) (Figure 2).

Importance of m^5C_{14} to the Mg^{2+}-Induced Structural Transition. Since the modified $tDNA^{Phe}_{AC}$ exhibited the Mg^{2+}-induced structural transition, but unmodified $tDNA^{Phe}_{AC}$ did not, one or more of the nucleosides within the only sequence difference between the two DNAs [$d(U_{13}m^5C_{14}U_{15})$ vs $d(T_{13}C_{14}T_{15})$, respectively] must be important for the structural change. In order to determine which of the nucleosides in the sequence $d(U_{13}m^5C_{14}U_{15})$ were important to the structural transition, three additional oligonucleotides were synthesized, $tDNA^{Phe}_{AC}$ $d(U_{13}m^5C_{14}T_{15})$, $tDNA^{Phe}_{AC}$ $d(U_{13}C_{14}U_{15})$, and $tDNA^{Phe}_{AC}$ $d(T_{13}m^5C_{14}U_{15})$ (Figure 1D–F), and studied by CD spectropolarimetry. In the absence of Mg^{2+}, these three oligonucleotides exhibited CD spectra very similar to each other and to that of the modified and unmodified $tRNA^{Phe}_{AC}$ molecules (results not shown). On the basis of these results, all five oligomers apparently have seven-membered loop structures in the absence of Mg^{2+} very similar to that found for modified $tDNA^{Phe}_{AC}$ under the same conditions (Guenther et al., 1992).

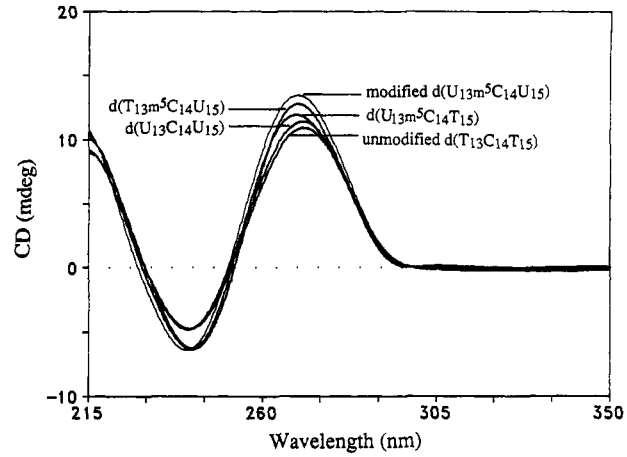

FIGURE 3: Circular dichroism spectra of the five $tDNA^{Phe}_{AC}$ analogs of $tRNA^{Phe}_{AC}$ in the presence of Mg^{2+}. The spectra of $tDNA^{Phe}_{AC}$ $d(U_{13}m^5C_{14}U_{15})$, $d(U_{13}m^5C_{14}T_{15})$, $d(U_{13}C_{14}U_{15})$, $d(T_{13}m^5C_{14}U_{15})$, and $d(T_{13}C_{14}T_{15})$ were obtained in 25 mM $MgCl_2$ and 10 mM sodium phosphate solution (pH 7) at 10 °C.

In the presence of 25 mM Mg^{2+}, the three partially modified $tDNA^{Phe}_{AC}$ molecules behaved quite differently from each other (Figure 3). All three oligonucleotides had Mg^{2+}-dependent structural transitions that could be measured by CD spectrometry. However, the samples differed considerably in the degree to which the CD wavelength maxima and minima and amplitudes changed. The largest difference, as previously reported (Guenther et al., 1992), was between spectra of the modified $tDNA^{Phe}_{AC}$ [with the three modified bases, $d(U_{13}m^5C_{14}U_{15})$, in the stem] and the unmodified $tDNA^{Phe}_{AC}$ (Figure 3).

With the addition of Mg^{2+}, the three partially modified $tDNA^{Phe}_{AC}$ samples exhibited CD spectral changes that were intermediate in wavelength and amplitude between those of the fully modified and unmodified tDNA. The $tDNA^{Phe}_{AC}$ $d(T_{13}m^5C_{14}U_{15})$ oligomer had a spectrum very close in wavelength maximum and minimum and amplitude to that of modified $tDNA^{Phe}_{AC}$ (Figure 3). The $tDNA^{Phe}_{AC}$ $d(U_{13}C_{14}U_{15})$ spectrum most closely resembled that of unmodified tDNA, whereas $tDNA^{Phe}_{AC}$ $d(U_{13}m^5C_{14}T_{15})$ had a spectrum that resembled $tDNA^{Phe}_{AC}$ $d(U_{13}m^5C_{14}U_{15})$ in wavelength shifts, but with similar amplitude to that of $tDNA^{Phe}_{AC}$ $d(U_{13}C_{14}U_{15})$. These results indicate that the modified deoxynucleoside m^5C_{14} is required for the Mg^{2+}-induced structural transition in $tDNA^{Phe}_{AC}$ and that dU_{15} is not necessary but enhances the transition.

Requirement for Complementary Bases in the Loop. With the addition of Mg^{2+}, the modified tDNA exhibited a structural transition that included the addition of two hydrogen bonds across what was a seven-membered loop, thus producing a two-base turn (Guenther et al., 1992). One of the two additional hydrogen bonds across the $tDNA^{Phe}_{AC}$ loop occurred between T_7 and A_{10}. By substituting an A for a T at position 7 in the modified $tDNA^{Phe}_{AC}$ sequence d(CCAGACA$_7$-GAAGAUm^5CUGG) [designated $tDNA^{Phe}_{AC}$ $d(A_7;U_{13}m^5C_{14}U_{15})$] the molecule may be able to bind Mg^{2+}, but will no longer form the Watson–Crick A·T base pair. The CD spectrum of $tDNA^{Phe}_{AC}$ $d(A_7;U_{13}m^5C_{14}U_{15})$ in the presence of 5 mM Mg^{2+} was similar to the spectrum in its absence, whereas the spectrum in the presence of 25 mM Mg^{2+} was also similar to that in the absence of Mg^{2+}, but had a slightly depressed amplitude at 268 nm (results not shown). Thus, no Mg^{2+}-dependent structural transition was evident in

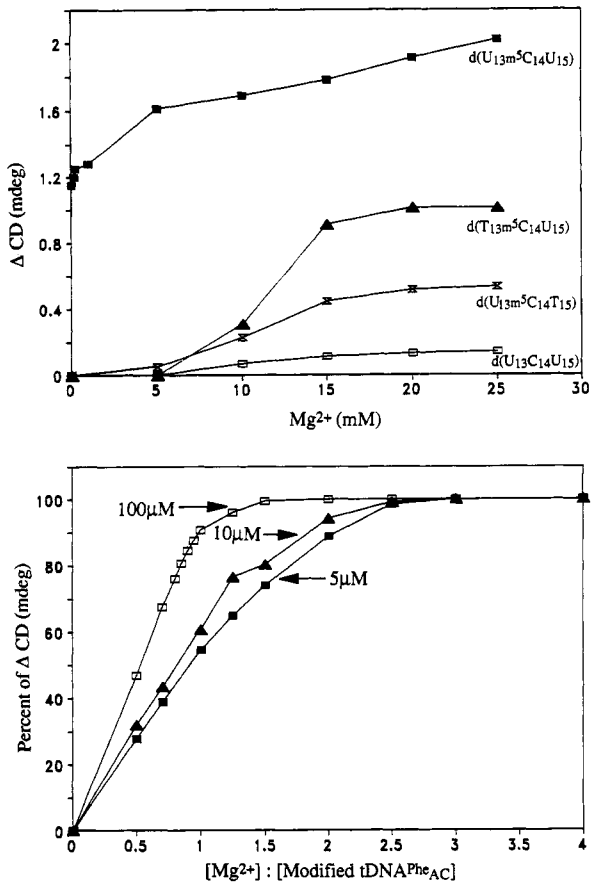

FIGURE 4: Mg^{2+} titration of tDNAs. The CD spectra of each of the five tDNAs were obtained at different concentrations (micromolar to millimolar) of Mg^{2+}. The change in amplitude [ΔCD (mdeg)] at 260 nm is plotted against the Mg^{2+} concentration. The final volumes and the absorbances of UV light at 260 nm of the titrated solutions were checked to make sure that there was no dilution or vaporization of the samples during the titration procedures. (A) The tDNAs $d(U_{13}m^5C_{14}U_{15})$, $d(T_{13}m^5C_{14}U_{15})$, $d(U_{13}m^5C_{14}T_{15})$, and $d(U_{13}C_{14}U_{15})$ were titrated with $MgCl_2$. The DNA concentration was 5 μM for all samples. The Mg^{2+} concentration was increased by increments of 2.5 μM until 10 μM (not all points are shown on the figure), followed by increments of 20 μM until 100 μM (not all points are shown on the figure), and finally by increments of 5 mM. The Mg^{2+} concentration was increased as the points on the curves indicate. (B) Titration of modified $tDNA^{Phe}_{AC}$ $d(U_{13}m^5C_{14}U_{15})$ with micromolar Mg^{2+}. The spectrum of tDNA $d(U_{13}m^5C_{14}U_{15})$ changed in response to micromolar concentrations of Mg^{2+} (panel A). Shown here is a detailed micromolar titration. The concentrations of the $tDNA^{Phe}_{AC}$ were 5, 10, and 100 μM as indicated on the figure. The Mg^{2+} concentration was increased relative to that of the tDNA from 0 Mg^{2+} to a Mg^{2+} to DNA ratio of 4:1. Changes in CD are reported as a percent of the maximum amplitude change.

the CD spectra of $tDNA^{Phe}_{AC}$ $d(A_7;U_{13}m^5C_{14}U_{15})$. Therefore, formation of the modified nucleoside-dependent, Mg^{2+}-induced conformation appears to require the T_7·A_{10} base pair as well.

Characterization of the Mg^{2+}-Induced Structural Transition. The amount of Mg^{2+} sufficient to induce a structural transition and stabilization of the $tDNA^{Phe}$ anticodon stem and loop structure was investigated. The four oligonucleotides that exhibited some change in CD spectra with the addition of Mg^{2+} were titrated with Mg^{2+} at 10 °C. The structural transitions were monitored in the CD spectra at the wavelength at which the largest differences between the $tDNA_{AC}$ oligomers would be expected, 260 nm. Results of these titrations are shown in Figure 4A. As expected from previous results (Figure 3), the modified $tDNA^{Phe}_{AC}$ $d(U_{13}m^5C_{14}U_{15})$ exhibited the largest amplitude change [ΔCD (mdeg)] as compared to the other tDNAs. All four tDNAs were at a concentration of 5 μM and exhibited structural transitions when millimolar concentrations of Mg^{2+} were added. However, in the titration of modified $tDNA^{Phe}_{AC}$ $d(U_{13}m^5C_{14}U_{15})$, we also observed a substantial change in the CD spectrum with the addition of only micromolar concentrations of Mg^{2+}. In Figure 4A this is seen as the early rising plateau for the Mg^{2+} titration curve with the modified tDNA. The results of a detailed analysis of the effect of micromolar concentrations of Mg^{2+} on the CD spectrum of modified $tDNA^{Phe}_{AC}$ are shown in Figure 4B. With the tDNA concentration held constant at 5, 10, and 100 μM, the Mg^{2+} concentration was varied to produce ratios of [Mg^{2+}]:[tDNA] from 0.5 to 4. Essentially the entire increase in amplitude exhibited in the early rising plateau of the Mg^{2+} titration curve of modified $tDNA^{Phe}_{AC}$ (Figure 4A) was achieved with a 1:1 ratio of Mg^{2+}:tDNA at a concentration of 100 μM (Figure 4B). The transitions observed with $tDNA^{Phe}_{AC}$ $d(U_{13}m^5C_{14}T_{15})$, $d(T_{13}m^5C_{14}U_{15})$, and $d(U_{13}C_{14}U_{15})$ were not completely induced until the Mg^{2+} concentration was >10 mM (Figure 4A). Therefore, the modified $tDNA^{Phe}_{AC}$ exhibited both micromolar and millimolar binding events with Mg^{2+}, whereas the other oligomers demonstrated only millimolar associations. These results indicate that when the entire $d(U_{13}m^5C_{14}U_{15})$ sequence is present, a micromolar Mg^{2+} binding is facilitated and the modified tDNA has both strong and weak Mg^{2+} binding sites. For the strong binding event, the association constant and binding stoichiometry of Mg^{2+} with the modified tDNA could be calculated by Scatchard analysis. The stoichiometry was 1 (with an error of ±0.05); i.e., a single strong Mg^{2+} binding site is indicated. From the K_d value of 1.09×10^{-6} M, the change in free energy ΔG for this Mg^{2+} binding event is −7.75 kcal/mol. The binding of Mg^{2+}, formation of hydrogen bonds between T_7 and A_{10} and/or between C_6 and G_{11}, stabilization of the A_5·dU_{13} base pair at the bottom of the stem, twisting of the sugar–phosphate backbone, base stacking, base bulging, or intercalation of the "bulged" A_{12}, and a change in the sugar puckering of the structure (Guenther et al., 1992) could all contribute to the change in free energy upon Mg^{2+} binding.

Biphasic Thermal Transition of m^5C_{14}-Containing $tDNA^{Phe}_{AC}$. Thermal denaturation of modified $tDNA^{Phe}_{AC}$ produced a biphasic UV melting curve with a low-temperature transition at 23 °C and a high-temperature transition at 52 °C (Guenther et al., 1992). Only the high-temperature transition was exhibited by unmodified $tDNA^{Phe}_{AC}$. Additional evidence for the Mg^{2+}-induced structural transition that occurs at low temperature and apparently only with oligomers that contain m^5C_{14} was obtained from results of UV- and CD-detected thermal transitions. All three $tDNA^{Phe}_{AC}$ samples containing m^5C_{14}, i.e., modified $tDNA^{Phe}_{AC}$ $d(U_{13}m^5C_{14}U_{15})$, $d(U_{13}m^5C_{14}T_{15})$, and $d(T_{13}m^5C_{14}U_{15})$, had UV-detected, biphasic thermal transitions in the presence of Mg^{2+} and T_m's of 23–24 and 51–53 °C (Table I). In contrast, unmodified $tDNA^{Phe}_{AC}$ $d(T_{13}C_{14}T_{15})$, $tDNA^{Phe}_{AC}$ $d(U_{13}C_{14}U_{15})$, and $tDNA^{Phe}_{AC}$ $d(A_7;U_{13}m^5C_{14}U_{15})$ had only monophasic transitions in the presence of Mg^{2+} with a T_m of ≈52 °C (Table I).

The biphasic melting of the Mg^{2+}-induced structure of modified $tDNA^{Phe}_{AC}$ $d(U_{13}m^5C_{14}U_{15})$ could be detected with CD spectropolarimetry (Guenther et al., 1992), as well as with UV spectroscopy. During the low-temperature thermal transition of the modified $tDNA^{Phe}_{AC}$, between 20 and 25 °C, the CD wavelength maximum shifted to longer wavelengths and decreased in amplitude, behavior opposite to that exhibited when Mg^{2+} was added to solutions of modified $tDNA^{Phe}_{AC}$

Table I: T_m's of Six Different tDNA$^{Phe}_{AC}$ Samples[a]

sample	T_m (°C)	
	5 mM Mg^{2+}	no Mg^{2+}
$d(U_{13}m^5C_{14}U_{15})$	23.2	52.8	47.0
$d(T_{13}m^5C_{14}U_{15})$	23.5	52.6	47.1
$d(U_{13}C_{14}U_{15})$	not obsd	51.8	46.8
$d(U_{13}m^5C_{14}T_{15})$	24.2	51.8	46.8
$d(T_{13}C_{14}T_{15})$	not obsd	52.0	46.5
$d(A_7;U_{13}m^5C_{14}U_{15})$	not obsd	51.6	45.8

[a] Samples in 10 mM sodium phosphate solution (pH 7), with or without 5 mM $MgCl_2$, were subjected to thermal denaturation and monitored by UV spectroscopy at 260 nm.

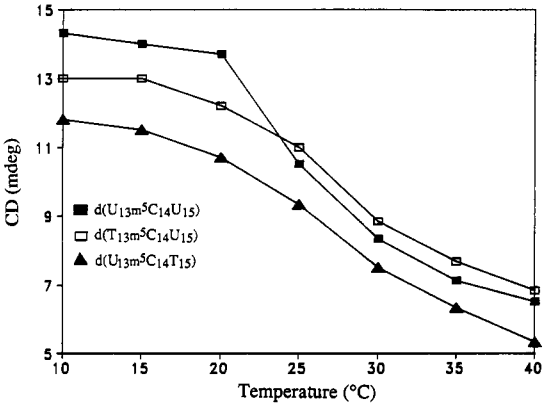

FIGURE 5: Low-temperature thermal denaturation of tDNAs containing m^5C. Thermal denaturation of the tDNA$^{Phe}_{AC}$ samples $d(U_{13}m^5C_{14}U_{15})$, $d(U_{13}m^5C_{14}T_{15})$, and $d(T_{13}m^5C_{14}U_{15})$ in the presence of Mg^{2+}. CD spectra were obtained at seven temperatures between 10 and 40 °C with the samples in 25 mM $MgCl_2$ and 10 mM sodium phosphate solution (pH 7). The amplitude (mdeg) at 260 nm is plotted versus temperature.

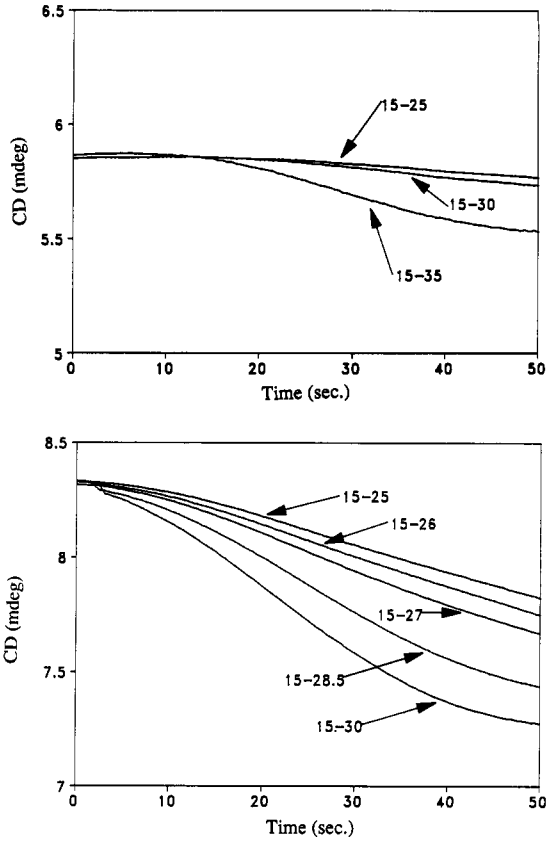

FIGURE 6: Rate of the low-temperature denaturation of modified tDNA$^{Phe}_{AC}$ $d(U_{13}m^5C_{14}U_{15})$. (A) Rate of thermal denaturation of modified tDNA$^{Phe}_{AC}$ $d(U_{13}m^5C_{14}U_{15})$ in the absence of Mg^{2+}. The samples in 10 mM sodium phosphate solution (pH 7) were equilibrated at 15 °C before the temperature was raised to 25, 30, or 35 °C. The circular dichroism was measured at 260 nm every 0.5 s after these temperature changes. The CD amplitude at 260 nm is plotted against time for each of the temperature jumps. (B) Rate of thermal denaturation of modified tDNA$^{Phe}_{AC}$ $d(U_{13}m^5C_{14}U_{15})$ in the presence of Mg^{2+}. The samples in 10 mM $MgCl_2$ and 10 mM sodium phosphate solution (pH 7) were equilibrated at 15 °C before the temperature was raised to 25, 26, 27, 28.5, or 30 °C. The circular dichroism was measured at 260 nm every 0.5 s after these temperature changes. The CD amplitude at 260 nm is plotted against time for each of the temperature jumps.

at 10 °C (Figure 2). Between 20 and 25 °C, the wavelength maximum shifted from 269 to 272 nm and its amplitude decreased by 40% of the total decrease exhibited between 10 and 40 °C. The Mg^{2+}-induced, low-temperature thermal transitions of the other two analogs containing m^5C_{14} could also be monitored by CD. Figure 5 demonstrates that between 20 and 30 °C the three m^5C_{14}-containing tDNA$^{Phe}_{AC}$ molecules had similar decreases in CD maxima at 260 nm. Therefore, the combined results of UV- and CD-monitored thermal transitions indicate that the Mg^{2+}-induced, low-temperature structural transition may be unique to the molecules containing m^5C_{14} and having the potential of forming a hydrogen bond between T_7 and A_{10}. Although micromolar Mg^{2+} binding to tDNA $d(T_{13}m^5C_{14}U_{15})$ and tDNA $d(U_{13}m^5C_{14}T_{15})$ could not be detected, these analogs did display low-temperature thermal transitions. Therefore, for these tDNAs the low-temperature thermal transition at 25 mM Mg^{2+} may constitute a melting of the same structure as that for tDNA $d(U_{13}m^5C_{14}U_{15})$, but one requiring higher concentrations of Mg^{2+} for comparable stabilization.

In order to analyze in detail the Mg^{2+}-induced, low-temperature structural transition, experiments were designed to determine the rate of thermal denaturation of the Mg^{2+}-induced structure. Three samples of the modified tDNA$^{Phe}_{AC}$ $d(U_{13}m^5C_{14}U_{15})$ without Mg^{2+} were equilibrated to 15 °C. Then, the temperature of each sample was raised to either 25, 30, or 35 °C. The CD spectrum began to change significantly only when the temperature was higher than 30 °C, and this change occurred within 30 s of the shift to 35 °C (Figure 6A). In the presence of Mg^{2+}, any CD-detected melting at temperatures below 30 °C would be due to the loss of the Mg^{2+}-induced structure [Guenther et al. (1992) and Figure 5]. Five samples of the modified tDNA$^{Phe}_{AC}$ with 10 mM Mg^{2+} were transferred from 15 °C to 25, 26, 27, 28.5, and 30 °C, respectively. The changes in amplitude of the CD maxima (260 nm) with time for these samples are shown in Figure 6B. The largest decrease in amplitude with time occurred between the 15 to 27 °C and 15 to 28.5 °C temperature shifts. For a pseudo-first-order reaction, the temperature-dependent reaction rate has the equation:

$$\frac{d[\text{reactant}]}{dt} = -k[\text{reactant}]$$

where k is the pseudo-first-order rate constant. The activation energy (E_a) for the Mg^{2+}-induced, low-temperature structural transition of modified tDNA$^{Phe}_{AC}$ was calculated using an Arrhenius plot and found to be 27.7 kcal/mol.

Some of the factors contributing to the stability of the Mg^{2+}-stabilized conformation might be determined by studying thermal denaturation of the molecule's base pairs by following exchange of the imino protons with the solvent as detected by NMR spectroscopy. Proton NMR spectra of modified tDNA$^{Phe}_{AC}$ in the presence of Mg^{2+} were collected at 12 temperatures between 10 and 40 °C. Five of these spectra

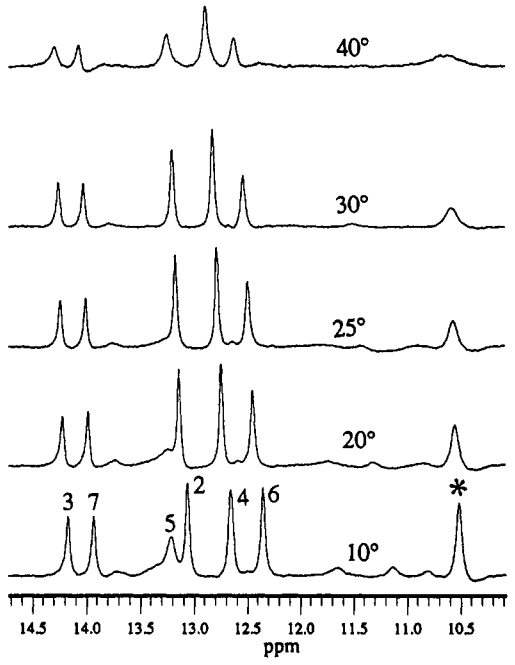

FIGURE 7: NMR spectra of tDNA$^{Phe}_{AC}$ d($U_{13}m^5C_{14}U_{15}$) taken at different temperatures. The NMR spectrum of modified tDNA$^{Phe}_{AC}$ in 10 mM $MgCl_2$ and sodium phosphate solution (pH 7) was taken at 12 different temperatures from 10 to 40 °C. The downfield portion of the spectrum, in which the imino proton resonances are found, is shown for five of the spectra, 10, 20, 25, 30, and 40 °C. The imino proton signals of the base pairs and of G_8 of tDNA$^{Phe}_{AC}$ d(U_{13}-$m^5C_{14}U_{15}$) in Mg^{2+} were already assigned (Guenther et al., 1992): 2, C_2·G_{16}; 3, A_3·dU_{15}; 4, G_4·m^5C_{15}; 5, A_5·dU_{13}; 6, C_6·G_{11}; 7, T_7·A_{10}; and *, G_8.

are shown in Figure 7. The resonance assigned (Guenther et al., 1992) to the imino proton of the A_5·dU_{13} base pair, signal 5, broadens and was lost before the temperature reached 25 °C. The imino resonance of the N-1 proton of G_8 (denoted as "*" in Figure 7) slowly broadens starting at the low temperature of 17 °C, but remains visible even at 40 °C. Interestingly, the imino resonances for the two hydrogen bonds formed across the seven-membered loop in the presence of Mg^{2+}, C_6·G_{11} signal 6 and T_7·A_{10} signal 7, remained essentially unchanged within the temperature range, 20–30 °C, at which the CD and UV spectra detect a low-temperature denaturation of the Mg^{2+}-induced structure. Only at the higher temperatures, 32–40 °C, do these resonances begin to exhibit significant broadening due to enhanced solvent exchange.

DISCUSSION

The Mg^{2+}-induced structural transition of the tDNA$^{Phe}_{AC}$ d($U_{13}m^5C_{14}U_{15}$) hairpin, a DNA analog of the yeast tRNA^{Phe} anticodon stem and loop, results in a double-stranded stem of five nucleotide complements held together by four base pairs and a seven-membered "loop" that is crossed by two hydrogen bonds producing a two-base turn and a single-base bulge (Guenther et al., 1992). We have determined, from CD and NMR studies, that the presence of the modified nucleoside m^5C_{14} supports this Mg^{2+}-induced structural transition. Nucleosides 13 and 15 are also implicated in the conformational change in that they facilitate formation of the new structure when these positions are dUs rather than Ts.

A- or B-Form DNA. The Mg^{2+}-induced conformational changes of tDNA$^{Phe}_{AC}$ d($U_{13}m^5C_{14}U_{15}$) are not simply a transformation from B- to A-form DNA. In fact, the CD spectra of modified tDNA$^{Phe}_{AC}$ d($U_{13}m^5C_{14}U_{15}$), or for that matter any of the tDNA$_{AC}$ samples, with or without Mg^{2+}, cannot be characterized as either B- or A-form DNA (Figure 2). With addition of Mg^{2+}, the CD spectrum of modified tDNA$^{Phe}_{AC}$ changes in the direction characteristic of A-form RNA. However, there is acknowledged difficulty in describing nucleic acid duplexes (Traver, 1992) and hairpins in solution relative to the standard B-form DNA and A-form RNA (Puglisi et al., 1990; Varani et al., 1991; Heus & Pardi, 1991). Consideration of counterions (Withka et al., 1992) and modifications (Agris & Sierzputowska-Gracz, 1990) seems to be essential, and the dynamic of the tDNA$^{Phe}_{AC}$ conformation is clearly both m^5C_{14}-dependent and Mg^{2+}-induced.

Features of the Mg^{2+}-Induced Conformation. The m^5C-dependent, Mg^{2+}-induced structural transition of tDNA$^{Phe}_{AC}$ d($U_{13}m^5C_{14}U_{15}$) occurs with a micromolar Mg^{2+} binding event and a ratio of Mg^{2+} to tDNA of 1:1. Thus, there is a single, strong Mg^{2+} binding site. In addition, there are weak (millimolar) binding sites. Our initial investigations of the DNA analog to the TΨC loop of yeast tRNA^{Phe} (S. Vallabha, R. H. Guenther, H. Sierzputowska-Gracz, and P. F. Agris, unpublished results) indicate that this 17-mer hairpin has only weak Mg^{2+} binding sites and they may be equivalent in number to the backbone phosphates. The presence of both strong and weak Mg^{2+} binding sites in DNA analogs of tRNA^{Phe} is consistent with investigations of the entire yeast tRNA^{Phe} structure (Jack et al., 1976, 1977; Quigley et al., 1978; Agris et al., 1985; Hyde & Reid, 1985; Reid & Cowan, 1990).

The divalent cation-induced structural transition in tDNA$^{Phe}_{AC}$ is characterized by formation of the A_5·dU_{13}, C_6·G_{11}, and T_7·A_{10} hydrogen bonds (Guenther et al., 1992) and a change in the location of the bulged A_{12} to a position that continues the 3′ stack after the two-base turn of the hairpin (H. Sierzputowska-Gracz and P. F. Agris, unpublished results). However, the A_5·dU_{13} base pair at the bottom of the tDNA$_{AC}$ stem is one of the features of the Mg^{2+}-stabilized structure that denature at low temperature (Figure 7). The A_{31}·Ψ_{39} base pair at the bottom of the anticodon stem of tRNA binds ions, and the hydrogen-bonded imino proton is temperature labile, indicating that the conformations of these tRNA nucleosides are easily altered (Hyde & Reid, 1985). Even so, pseudouridine is considerably more versatile in its hydrogen bonding interaction than U and, therefore, relative to U is capable of stabilizing hydrogen bonding at the bottom of the anticodon stem (Davis & Poulter, 1991). Another feature of the Mg^{2+}-induced tDNA$_{AC}$ structure that may be altered at low temperature is the base-stacked, bulged A_{12}. The structural change produced by a bulge in a nucleic acid helix may be propagated for several base pairs, and bulges may increase the number of conformations that a helix may take, though this is thought to be more restricted in the case of DNA (White & Draper, 1989). Therefore, the ΔG of –7.75 kcal/mol calculated for formation of the Mg^{2+}-induced structure could be associated with stabilization of the A_5·U_{13} base pair and the positioning of the bulged A_{12}, including its base-stacking interactions.

Location and Coordination of the Mg^{2+}. The location of the strong Mg^{2+} binding site in tDNA$_{AC}$ can be approximated from a comparison of the Mg^{2+}-induced low-temperature thermal transitions monitored by CD (Figures 5 and 6) and NMR (Figure 7). The broadening and disappearance of the NMR signal assigned to the imino proton of the A_5·U_{13} base pair at the bottom of the stem occurred at the temperature (≈26 °C) that the Mg^{2+}-induced conformation is lost from the CD spectrum, yet the C_6·G_{11} and T_7·A_{10} base pairs are

stable at this temperature. Therefore, we place the magnesium in the upper part of the hairpin loop near the A_5-dU_{13} base pair and the bulged A_{12}. Four distinct magnesium ions have been found in the crystal structure of yeast $tRNA^{Phe}$; one is in the upper part of the anticodon loop (Quigley et al., 1978). The conformation of the anticodon stem/loop domain within the tRNA molecule is stabilized by coordination of the Mg^{2+} cation. The ion located in the upper part of the anticodon loop is coordinated to the phosphate oxygen of Y_{37}, a derivative of G, and the oxygens contributed by five water molecules. The water molecules are hydrogen bonded to the base atoms on Cm_{32}, which is 2′-O-methylated and thereby prevented from using the 2′-O for Mg^{2+} coordination, Y_{37}, A_{38}, and Ψ_{39}. Thus, an inner-sphere Mg^{2+} coordination site forms that does not depend on 2′-OH groups. Therefore, the strong Mg^{2+} binding site in the upper part of the $tDNA_{AC}$ loop could easily resemble the site in tRNA by including the phosphate of G_{11} and, through water, the bases of C_6, G_{11}, A_{12}, and dU_{13}. In contrast, specific 2′-OHs are necessary, or important, for Mg^{2+} coordination and Mg^{2+}-facilitated function of the ribozyme and RNase P (Perreault & Altman, 1992; Perreault et al., 1991).

Contributions of Nucleoside Modification and Mg^{2+} to Structure, Dynamics, and Function. The Mg^{2+} coordination sites in the upper parts of the tRNA anticodon loop, and the tDNA analog of the anticodon, are somewhat distant from their respective m^5C_{40} and $d(m^5C_{14})$, yet the methylated C seems necessary for the production of the single, strong Mg^{2+} binding site. The contributions of nucleoside modifications to stem/loop conformation, dynamics, and ion binding in DNA and RNA have not been adequately investigated. The major base sequence of the family of molecules used in this study is identical to that of yeast $tRNA^{Phe}_{AC}$. However, a tRNA molecule devoid of modified nucleosides and synthesized using T7 RNA polymerase was found to require higher than expected concentrations of Mg^{2+} (50 mM) before the ^1H NMR spectrum approached that of the native molecule (Hall et al., 1989). In the absence of Mg^{2+} and at low Mg^{2+} concentrations, fully modified *Escherichia coli* and yeast $tRNA^{Phe}$ have been shown to exist as a mixture of two or more conformations in slow exchange (Kopper et al., 1983; Hyde & Reid, 1985; Agris et al., 1986). The "melting" temperature of the unmodified $tRNA^{Phe}$ at concentrations of $MgCl_2$ >5 mM was still 5 °C lower than occurred with the native form (Sampson & Uhlenbeck, 1988). The structure and conformation of fully modified yeast $tRNA^{Phe}$ and the unmodified T7 transcript of yeast $tRNA^{Phe}$ were investigated via strand cleavage with the photoactivated reagent $Rh(phen)_2phi^{3+}$ (Chow et al., 1992). The cleavage pattern of the unmodified T7 transcript was similar to that of the mature tRNA except for additional sites occurring in the anticodon loop and stem of the transcript: C_{27}, A_{36}, G_{37}, and A_{38}. Cleavage of the unmodified transcript was enhanced in 10 mM $MgCl_2$, whereas increasing concentrations of Mg^{2+} decreased cleavage of the anticodon of fully modified $tRNA^{Phe}$ at Y_{37}, A_{38}, and Ψ_{39}. These studies suggest that localized conformational changes occur in the anticodon with increasing Mg^{2+}, that they occur exactly at the known site of Mg^{2+} binding, and that Mg^{2+} and modified nucleosides contribute all to the stability of the native structure.

Thus, the anticodon of a tRNA may have more than a single conformation depending upon modification(s), divalent cation binding, and other intramolecular functional interactions. Since some of the yeast $tRNA^{Phe}$ identity elements for specific interaction with its cognate aminoacyl-tRNA synthetase are within the anticodon loop (Sampson et al., 1992), an easily converted loop conformation could enhance specificity and be important to subsequent interactions with elongation factor and the ribosome. Methylation of cytosine-14 in the yeast $tDNA^{Phe}_{AC}$ enabled the molecule to form more than one conformation. By promoting a Mg^{2+}-induced conformational transition that is labile at low temperatures, m^5C imparted to the nucleic acid the ability to enter a dynamic pathway not otherwise available under physiological conditions.

In summary, these studies demonstrate that modified nucleosides, and not just the sequence of major bases alone, can affect the binding of Mg^{2+} and subsequent conformational changes that could then modulate the functions of tRNAs. Independent of 2′-OHs, the single strong, m^5C-dependent Mg^{2+} binding site in $tDNA^{Phe}_{AC}$ determines the conformation of the anticodon loop. We conclude that a single modification, base methylation of cytosine in the anticodon stem (position 40 in $tRNA^{Phe}$), plays a potentially critical role in function by facilitating Mg^{2+} binding, defining a new local conformation, and changing the global structure of the tRNA anticodon loop.

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SUPPLEMENTARY MATERIAL AVAILABLE

Figures showing the following: CD spectra of $tDNA^{Phe}_{AC}$ $d(U_{13}m^5C_{14}U_{15})$, $d(T_{13}m^5C_{14}U_{15})$, $d(U_{13}m^5C_{14}T_{15})$, $d(U_{13}C_{14}U_{15})$, and $d(T_{13}C_{14}T_{15})$ without Mg^{2+}; CD spectra of $tDNA^{Phe}_{AC}$ $d(A_7;U_{13}m^5C_{14}U_{15})$ in the presence of 5 and 25 mM Mg^{2+} and in its absence; Scatchard analysis of strong Mg^{2+} binding to $tDNA^{Phe}_{AC}$ $d(U_{13}m^5C_{14}U_{15})$; and Arrhenius plot from thermal denaturation of the Mg^{2+}-induced conformational transition (4 pages). Ordering information is given on any current masthead page.

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