if not all, chemotherapeutic drugs which act through topoisomerase II will display a similar ability to interfere with the enzyme's religation event.

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Lead-Catalyzed Cleavage of Yeast tRNA^{Phe} Mutants†

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ABSTRACT: Yeast tRNA^{Phe} lacking modified nucleotides undergoes lead-catalyzed cleavage between nucleotides U17 and G18 at a rate very similar to that of its fully modified counterpart. The rates of cleavage for 28 tRNA^{Phe} mutants were determined to define the structural requirements of this reaction. The cleavage rate was found to be very dependent on the identity and correct positioning of the two lead-coordinating pyrimidines defined by X-ray crystallography. Nucleotide changes that disrupted the tertiary interactions of tRNA^{Phe} reduced the rate of cleavage even when they were distant from the lead binding pocket. However, nucleotide changes designed to maintain tertiary interactions showed normal rates of cleavage, thereby making the reaction a useful probe for tRNA^{Phe} structure. Certain mutants resulted in the enhancement of cleavage at a "cryptic" site at C48. The sequences of *Escherichia coli* tRNA^{Phe} and yeast tRNA^{Arg} were altered such that they acquired the ability to cleave at U17, confirming our understanding of the structural requirements for cleavage. This mutagenic analysis of the lead cleavage domain provides a useful guide for similar analysis of autocatalytic self-cleavage reactions.

Certain ions, including Pb^{2+}, Zn^{2+}, and Eu^{3+}, are capable of cleaving purified tRNAs at precise locations to give 2′,3′ cyclic phosphate and 5′ hydroxyl termini (Rordorf et al., 1976; Werner et al., 1976; Ciesiolka et al., 1986). The best studied example of this phenomena is the lead-induced cleavage of yeast tRNA^{Phe} between residues D17 and G18 (Dirheimer et al., 1972; Werner et al., 1976; Krzyzosiak et al., 1988). X-ray diffraction studies on the cleaved and uncleaved tRNA^{Phe}–lead complex implicated one of the three tightly bound lead ions in the cleavage reaction (Brown et al., 1983, 1985; Ruben & Sundaralingam, 1983; Sundaralingam et al., 1984). This lead ion, termed Pb(1), is precisely coordinated in a pocket formed

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by eight residues in the D and T loops in such a way that it can remove the proton from the 2′ hydroxyl of ribose 17. Brown et al. (1985) propose that the resulting 2′ oxygen nucleophile can then attack phosphate 17 to give a pentacovalent phosphorus intermediate which decays to form the 2′,3′ cyclic phosphate and, after protonation, the 5′ hydroxyl. Since the pH optimum for cleavage was found to be about 7, it appeared likely that the titrated form of lead, $Pb(OH)^+$, was the active species. The ability of $Pb(OH)^+$ to promote nonspecific cleavage of RNA has been known for many years (Britten, 1962; Farkas, 1968). As in many metalloenzymes, the high specificity for cleavage of $tRNA^{Phe}$ can be understood in terms of the precise orientation of the active metal ion with respect to the cleavage site. Little if any cleavage of $tRNA^{Phe}$ is observed near the location of two other tightly bound lead ions which are presumably not as favorably oriented.

Although it is not known to have biological relevance, the lead-induced cleavage of yeast $tRNA^{Phe}$ has been considered a model for certain types of autocatalytic cleavage of RNA (Brown et al., 1983; Cech, 1987; Sampson et al., 1987). As part of their replication pathway, the genomes of hepatitis delta RNA and certain plant satellite RNAs also cleave at a unique site to give 2′,3′ cyclic phosphate and 5′ hydroxyl termini (Buzayan et al., 1986a,b; Hutchins et al., 1986; Kuo et al., 1988). Truncation experiments indicate that a relatively small portion of RNA genome is needed for self-cleavage (Buzayan et al., 1986a,b, Forster & Symons, 1987). The best studied "hammerhead" domain consists of about 40 nucleotides containing 3 helices and 13 conserved nucleotides. While there is no evidence that lead is needed for the hammerhead cleavage reaction, its cleavage rate is stimulated by the presence of divalent cations (Hutchins et al., 1986; Uhlenbeck, 1987).

We have used in vitro transcription with T7 RNA polymerase to prepare an RNA molecule having the sequence of yeast $tRNA^{Phe}$ but lacking the modified nucleotides (Sampson & Uhlenbeck, 1988). The ability of the transcript to be aminoacylated by yeast phenylalanyl-tRNA synthetase with virtually normal kinetics suggests that it is folded in a structure very similar to that of native $tRNA^{Phe}$. This is supported by proton NMR experiments (Hall et al., 1989). By preparing numerous mutants of the sequence, we have exploited this system to deduce the nucleotides in the $tRNA^{Phe}$ sequence that are used by the cognate synthetase to distinguish $tRNA^{Phe}$ from other tRNAs in the cell (Sampson et al., 1989a).

In this work we compare the lead cleavage properties of the unmodified $tRNA^{Phe}$ transcript with those of native yeast $tRNA^{Phe}$. In addition, the rate of lead cleavage of a substantial number of mutants of the $tRNA^{Phe}$ sequence will be determined. It will be shown that lead cleavage can serve as a useful independent criteria for the overall folding of $tRNA^{Phe}$. This work also represents the first thorough mutagenic analysis of a metal ion binding pocket in nucleic acids. Since the X-ray crystal structure of $tRNA^{Phe}$ is known, this analysis should be valuable in interpreting similar mutagenic studies in other catalytic RNAs.

Materials and Methods

tRNAs. Plasmids containing mutants of the yeast $tRNA^{Phe}$ gene flanked by the T7 RNA polymerase promoter and a *Bst*N1 restriction site were prepared from synthetic oligonucleotides by procedures described previously (Sampson & Uhlenbeck, 1988; Sampson et al., 1989).

tRNA transcripts were obtained by runoff transcription of *Bst*N1 cleaved plasmid in 100-μL reaction mixtures that contained 10 μg of DNA, 1 mM of each NTP, 5 mM dithiothreitol, 6 mM $MgCl_2$, 1 mM spermidine, 40 mM Tris-HCl (pH 8.1), 50 μg/mL bovine serum albumin, and 80 μg/mL T7 RNA polymerase. Transcripts were either internally labeled with [α-^{32}P]CTP at a specific activity of 100 Ci/mol or 5′ end labeled with [γ-^{32}P]GTP at a specific activity of 1250 Ci/mol. After incubation at 42 °C for 1 h, the reaction mixtures were diluted with an equal volume of 7 M urea and dyes and loaded on a 15% denaturing polyacrylamide gel. After electrophoresis, the tRNAs were located by autoradiography, excised, and eluted from the gel in a buffer containing 0.3 M sodium acetate, 1.6 M NaCl, and 10 mM Tris-HCl (pH 7.0). The eluted RNAs were ethanol precipitated twice to remove all traces of EDTA and stored in 10 mM Tris-HCl (pH 8.0).

Native yeast $tRNA^{Phe}$ (Boehringer-Mannheim) and the unmodified transcript were 3′ end labeled with (5′-^{32}P)pCp (England & Uhlenbeck, 1978) or 5′ end labeled by dephosphorylation with alkaline phosphatase followed by phosphorylation with [γ-^{32}P]ATP and polynucleotide kinase.

Cleavage Reactions. Lead acetate stocks (10 mM) were stored at −70 °C to avoid formation of an insoluble lead carbonate species. All tRNAs were renatured by heating to 70 °C for 1 min in the storage buffer and slow cooled to room temperature prior to use. A typical 40-μL cleavage reaction contained 2 μM tRNA (approximately 100 nCi), 15 mM morpholinopropanesulfonic acid (Mops) (pH 7.0), 1.5 mM spermine, 15 mM $MgCl_2$, and 200 μM $Pb(OAc)_2$. The reactions were initiated by the addition of $Pb(OAc)_2$ after the remaining components had equilibrated at the reaction temperature (typically 25 °C). At appropriate intervals 5-μL aliquots were removed and added to 5 μL of 7 M urea, 50 mM EDTA, and dyes. The cleavage reactions were analyzed on 15% polyacrylamide/7 M urea gels. The full-length tRNA and all defined cleavage products were located by autoradiography, excised from the gel, and Cerenkov counted.

For mapping the cleavage site, 10-μL reactions contained 0.5 μCi of end-labeled RNA and 50 μg of tRNA carrier. Lead cleavage was carried out as described above with a 10-min incubation at 40 °C. The T1 digestion was carried out with 2 milliunits of RNase T1 in 10 mM Tris-HCl and 1 mM EDTA (pH 8.0) for 15 min at 50 °C. Base hydrolysis was achieved in 50 mM glycine hydrochloride (pH 9.0) at 95 °C for 15 min.

Results

Cleavage of Unmodified tRNA. The sites of lead cleavage in the $tRNA^{Phe}$ transcript were determined by using a 5′ ^{32}P labeled transcript (Figure 1A). Comparison of the lead cleavage lane with the partial base hydrolysis and T1 digestion lanes on a sequencing gel located the major cleavage site after U17. This corresponds to the D17 cleavage site reported previously for fully modified $tRNA^{Phe}$ (Dirheimer et al., 1972). It is interesting to note that lead cleavage is not entirely specific. Minor products are observed corresponding to cleavage after U16 and G15. Lead cleavage of a homogeneously labeled transcript reveals an additional minor site at C48. Although the U16 and G15 sites could result from simultaneous or subsequent cleavage by the same tightly bound lead, the lead atom responsible for the rather distant C48 cleavage site is unclear.

The kinetics of lead cleavage of 3′ end labeled $tRNA^{Phe}$ transcript and fully modified $tRNA^{Phe}$ are compared in Figure 1B. Although inspection of the autoradiogram suggests that cleavage is quite specific throughout the reaction, longer exposures indicate that many other products accumulate at longer incubation times that presumably are the result of additional nonspecific cleavage events which occur at lower

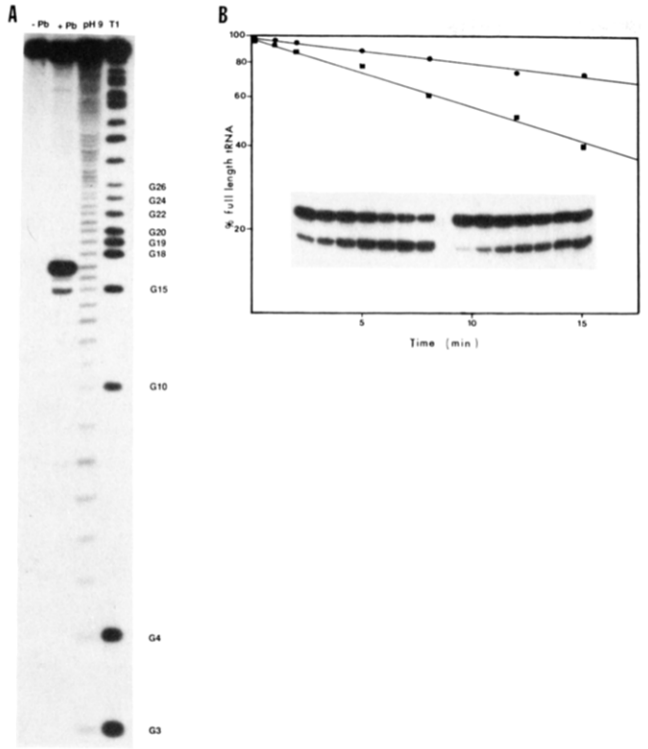

FIGURE 1: Lead cleavage of tRNA^{Phe}. (A) Location of the cleavage site determined by using 5′ ^{32}P labeled tRNA^{Phe} transcript. The lanes on the sequencing gel are the transcript untreated (−Pb), reacted with lead (+Pb), partially hydrolyzed with alkali (pH 9), and partially digested with RNase T1 (T1). (B) Cleavage kinetics of 3′ ^{32}P labeled native yeast tRNA^{Phe} (■) and tRNA^{Phe} transcript (●) in 200 μM $Pb(OAc)_2$, 15 mM $MgCl_2$, 1.5 mM spermine, and 15 mM Mops, pH 7.0 at 25 °C. (Insert) Autoradiogram of the polyacrylamide gel showing cleavage kinetics: (left) native yeast tRNA^{Phe}; (right) tRNA^{Phe} transcript.

rates at many locations in the molecule. Since first-order kinetics are only observed at low extents of cleavage, cleavage rates are determined by using data from the first 20% of the reaction. Even though the tRNA^{Phe} transcript lacks all 14 modified nucleotides, it exhibits a cleavage rate only 2-fold lower than the fully modified tRNA. This clearly indicates that the unmodified transcript can be used to study the lead cleavage reaction in detail.

The cleavage properties of the native tRNA and the transcript were further compared by determining the rate of reaction as a function of lead concentration. The lead ion responsible for the cleavage of tRNA^{Phe} is bound to a site that is only 2 Å away from one of the known magnesium binding sites (Brown et al., 1985; Jack et al., 1977). Since the two ions share several coordinating atoms, it is not possible for both ions to bind the tRNA simultaneously. The increase in the cleavage rate with increasing lead concentration observed for both RNAs (Figure 2A) is consistent with competition between the two ions. In 15 mM $MgCl_2$ buffer, it was not possible to obtain accurate data at lead concentrations greater than 0.5 mM due to the high rate of cleavage and the large proportion of nonspecific cleavage events. Since no indication of saturation was observed under the conditions tested, it was not possible to determine the relative binding constants of the two ions. It is therefore unclear whether the 2-fold greater cleavage rate observed for the native tRNA at all lead concentrations is a consequence of a difference in ion binding affinities resulting in an increased proportion of bound lead or the result of a structure more susceptible to cleavage.

As shown in Figure 2B, the cleavage rates at 200 μM $Pb(OAc)_2$ vary as a function of magnesium concentration in a

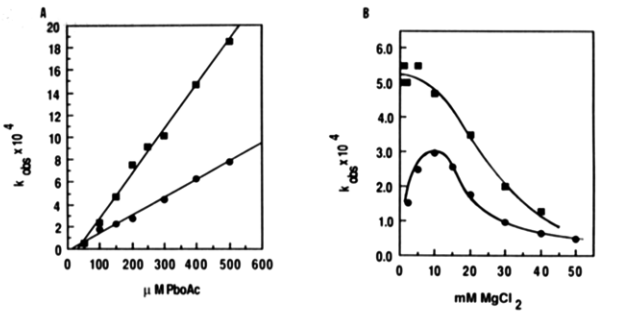

FIGURE 2: Divalent ion dependence of cleavage. (A) Cleavage rate of 3′ ^{32}P labeled tRNA^{Phe} (■) and transcript (●) in 15 mM $MgCl_2$, 1.5 mM spermine, and 15 mM Mops, pH 7.0 at 25 °C as a function of $Pb(OAc)_2$ concentration. (B) Cleavage rates at 200 μM $Pb(OAc)_2$, 1.5 mM spermine, and 15 mM Mops, pH 7.0 at 25 °C, as a function of $MgCl_2$ concentration.

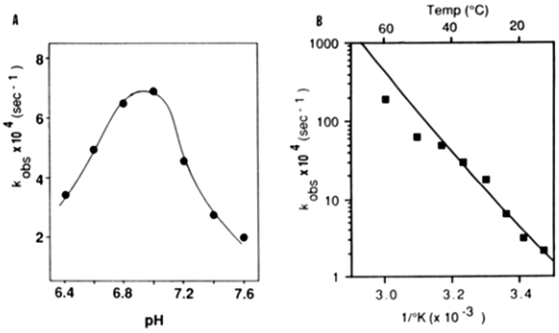

FIGURE 3: Lead cleavage of tRNA^{Phe} transcript. (A) pH–rate profile in 400 μM $Pb(OAc)_2$, 15 mM $MgCl_2$, 1.5 mM spermine, and 15 mM Mops, pH 6.4–7.6 at 25 °C. (B) Arrhenius plot at pH 7.0 under the above conditions.

complex manner that is different for the two tRNAs. Above 10 mM $MgCl_2$ the rates decrease with increasing $MgCl_2$ for both tRNAs in an exponential manner as expected for competition between the two ions. Under these conditions, the native tRNA is cleaved twice as rapidly as is the transcript. However, at lower $MgCl_2$ concentrations the cleavage rate of the native tRNA becomes constant, while the cleavage rate of the transcript actually decreases with decreasing magnesium concentration. It is likely that these deviations from simple competition between the two ions are a consequence of an altered structure of the tRNA at low magnesium concentrations. Although a difference in the conformation of native tRNA^{Phe} between low and high magnesium concentrations has been detected by many methods (Potts et al., 1981), the structure of the low-magnesium form remains unknown. Presumably the precise orientation between the T loop and the D loop required for cleavage is altered at the low magnesium concentration. Structural studies support the view that the transcript adopts an alternate conformation at higher magnesium concentrations than the native tRNA. The transcript has a lower T_m and a broader melting profile than the native tRNA^{Phe} at every magnesium concentration (Sampson & Uhlenbeck, 1988). NMR data also show an altered secondary and tertiary structure of the transcript at magnesium concentrations where the modified tRNA^{Phe} remains native (Hall et al., 1989).

Specific cleavage of the tRNA molecule by lead is highly pH dependent. The bell-shaped curve with an optimum at pH 7.0 (Figure 3A) can be explained by two effects. At low pH the lead ion binds to the tRNA at the same sites as at higher pH, but no cleavage at Pb(1) is observed (Brown et al., 1985). The increase in cleavage rate with pH is consistent with the proposal that $Pb(OH)^+$ is the active species for cleavage

Table I: Rate of Lead Cleavage at U_{17} for $tRNA^{Phe}$ Transcripts with Mutations near Pb(1)[a]

residue	atoms and (distance) to Pb(1)[b]	mutation	k_{obs} ($s^{-1} \times 10^4$)	rel k_{obs}[c]
U59	O4 (2.2), N3 (3.6) O2 (5.5), N1 (5.6)	C59	0.7	0.10
C60	N3 (2.8), N4 (3.5) O2 (3.6), N1 (4.9), O1′ (6.7)	U60	0.6	0.08
G20	O6 (4.4), N7 (5.0) N1 (6.7), OL (6.8)	U20 A20	6.6 6.1	0.92 0.85
G15 (paired with C48)	O2′ (5.3), N2 (6.2) N3 (6.2)	A15 U48 G48 A15, U48	6.1 3.0 1.5 8.6	0.85 0.42 0.21 1.19
U17	O2′ (6.0), OR (6.8)	C16, C17	9.6	1.33
U16	OL (4.8), O1′ (5.0), P (6.0) O3′ (6.1), O2 (6.2), N1 (6.3) O5′ (6.3), N3 (6.4)			
G18 (paired with U55)	O5′ (5.9), O1′ (6.3)	A18 C55 A55 A18, C55	3.2 3.1[d] 0.7[d] 3.0	0.44 0.43 0.10 0.42
G19 (paired with C56)	OR (3.4), P (4.9), O3′ (5.6) O5′ (5.7), OL (5.8)	C19 G56 C19, G56 C19, G56, C59	0.7[d] 0.5[d] 6.9 0.2[d]	0.10 0.07 0.96 0.03

[a] All reactions in 400 μM $Pb(OAc)_2$, 15 mM $MgCl_2$, 1.5 mM spermine, and 15 mM Mops, pH 7.0 at 25 °C. [b] Taken from Brown et al. (1985). [c] Relative to the wild-type $tRNA^{Phe}$ transcript $k_{obs} = 7.2 \times 10^{-4}$ s^{-1}. [d] Cleavage at C48 seen as well, see Table III.

(Brown et al., 1985) since lead has a pK of 7.5 (Baes & Mesmer, 1976). At higher pH values the cleavage rate decreases due to the lower solubility of lead ions by the formation of polyhydroxo species (Baes & Mesmer, 1976).

The rate of lead cleavage of the transcript as a function of temperature is shown in the form of an Arrhenius plot in Figure 3B. The plot is linear between 4 and 42 °C above which temperature it deviates from linearity. Data cannot be obtained above 60 °C since specific cleavage is no longer observed. The absorbance–temperature profile of the transcript in 8 mM $MgCl_2$ (without lead) shows virtually no change in absorbance until 65 °C followed by a sharp transition above 75 °C (Sampson & Uhlenbeck, 1988). Thus, the deviation from linearity of the Arrhenius plot determined in 15 mM $MgCl_2$ is unlikely to be the result of a global melting of the tertiary structure, but rather a more subtle structural change or a reduction in the affinity of the lead ion.

Mutations in the Lead Binding Pocket. The crystallographic data locate the Pb(1) site within 7 Å of 33 atoms associated with 8 residues in the D loop and T loop (Brown et al., 1985; Figure 4). Pb(1) lies close to nucleotide functional groups of four residues (G15, G20, U59, and C60), making them attractive candidates for mutagenesis. The phosphodiester backbone of the other four residues (U16, U17, G18, and G19) makes up the remainder of the pocket and contains the substrate of the cleavage reaction. Although the crystallographic resolution was not sufficient to determine the exact coordination of the lead ion, the close proximity of Pb(1) to the O4 of U59 (2.2 Å) and the N3 of C60 (2.8 Å) suggest direct coordination, whereas atoms slightly further away such as the O6 of G20 (4.4 Å) could be coordinated through H_2O (Brown et al., 1985).

Table I gives the rates of lead cleavage for transcripts with mutations in the lead binding pocket as well as the distances of various atoms to Pb(1). Conservative mutations in the two pyrimidines that are the closest to Pb(1) have a substantial effect on the cleavage rate. Both U59C and C60U cleave at U17 with the same specificity, but their cleavage rates are reduced about 10-fold in each case. While both U59 and C60 are stacked within the tertiary structure, their substitution with

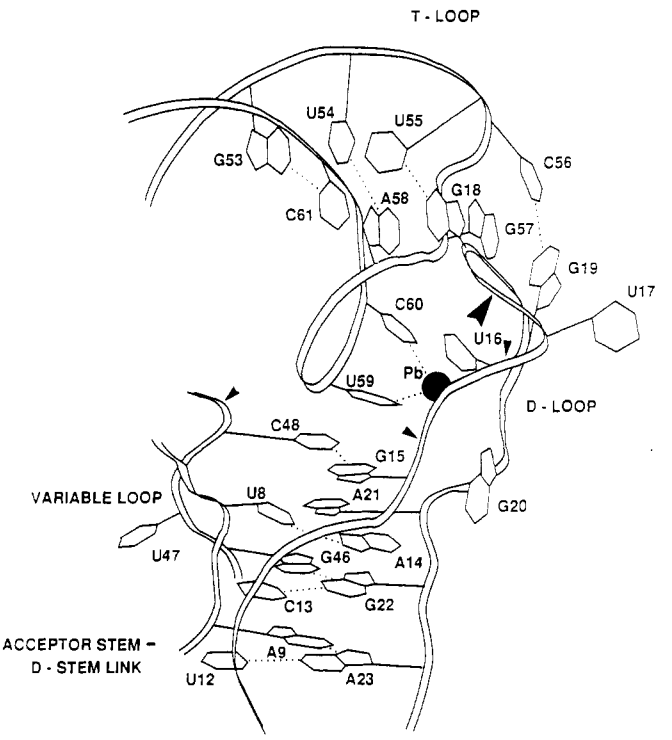

FIGURE 4: Lead cleavage domain. Diagram adapted from the crystal structure where the phosphodiester backbone is represented by a ribbon and the functional groups are omitted from the purine and pyrimidine rings. The solid circle indicates the position of Pb(1). Dotted lines indicate the proposed coordination between lead and U59–C60 as well as pairing between residues, but are not intended to represent actual bonds. The major site of lead cleavage is represented by the large arrowhead near residue 17. Minor cleavage sites are represented by small arrowheads.

the other pyrimidine would not be expected to disrupt the folding of the tRNA. This is supported by the fact that these same two mutants aminoacylate normally with yeast phenylalanyl tRNA synthetase (J. Sampson, unpublished experiments). Thus, the most likely interpretation for the slow cleavage of the C59 and U60 mutants is that either they show a reduced affinity for lead or the position of the bound lead is altered in such a way that cleavage is less efficient. Attempts to increase the cleavage rate for either mutant relative to the wild type by raising the $Pb(OAc)_2$ concentration were unsuccessful.

The single-stranded guanosine at position 20 lies somewhat further away from the Pb(1) site but still has the potential for coordination with lead. In addition, G20 is important for recognition of tRNA^{Phe} by its cognate synthetase (Sampson & Uhlenbeck, 1988). Two different mutations at this position have virtually no effect on the lead cleavage rate. Although the G20A change might not greatly alter the interaction with lead due to the maintenance of a potential contact at N7, the G20U mutation would be expected to alter the cleavage rate if functional groups on G20 contributed substantially to the formation of the lead binding pocket. Since this is not the case, it can be concluded that G20 is not directly involved in the lead cleavage reaction.

Mutation of G15 is potentially complicated by the fact that it forms a trans base pair with C48. The decrease in cleavage rate observed when C48 is substituted with either U or G (Table I) suggests that disrupting the tertiary base pair destabilizes the tRNA structure near the Pb(1) binding site. However, it appears unlikely that G15 interacts directly with Pb(1) since the mutation G15A remains active in cleavage either as a single mutant or as a double mutant with C48U. Although an A15–U48 tertiary base pair is the only alternative that occurs naturally (Levitt, 1969), model building suggests that an A15–C48 trans pair is also possible without a major distortion of the phosphodiester backbone (Sampson et al., 1990). Thus, as long as the tRNA backbone is maintained in the correct configuration by this tertiary interaction, the identity of nucleotide 15 is not important for the lead cleavage reaction.

The phosphodiester backbone of residues U16 and U17 is about 5–6 Å away from Pb(1). This places the 2′ hydroxyl of ribose 17 approximately the correct distance from the active $Pb(OH)^+$ (Brown et al., 1985). Since this region of the molecule is quite flexible, some movement may be required to attain the correct orientation for cleavage. Since the nucleotide bases of U16 and U17 are pointed away from Pb(1), it is not surprising that their mutation to C residues did not alter the cleavage rate. The same residues are dihydrouridine in native tRNA^{Phe}.

The conserved G18, 5′ to the cleavage site, interacts via a single hydrogen bond with the conserved U55 in the T loop. Since all the atoms in G18 are 6 Å or more away from Pb(1), one does not expect them to contribute substantially to the lead binding pocket. The observed decrease in the rate of lead cleavage for mutations in either G18 or U55 is presumably a result of destabilizing the folding of the tRNA structure.

The third closest atom to Pb(1) is OR of phosphate 19. Residue 19 is a guanosine that forms a slightly distorted Watson–Crick base pair with cytosine 56. Both the G19C and C56G mutants which disrupt the base pair result in a 5-fold decrease in the lead cleavage rate. The G19C, C56G double mutant which restores the base pair shows a normal rate of cleavage. These experiments clearly indicate that when this tertiary interaction is disrupted, the lead binding pocket is altered in such a way that cleavage is impaired. This could be the result of either weaker binding of lead due to the loss of the contact at OR19 or destabilization of the backbone structure near the cleavage site.

Table II: Rate of Lead Cleavage at U_{17} for tRNA^{Phe} Transcripts with Mutations Further from Pb(1)[a]

interaction	mutation	k_{obs} ($s^{-1} \times 10^4$)	rel k_{obs}[b]
U8–A14	A8	1.8	0.25
	C8	0.7	0.10
	G14	2.2	0.31
U54–A58	A54	0.4[c]	0.06
	C54	1.4	0.19
	G58	1.8[c]	0.25
G53–C61	C53–G61	0.6[c]	0.08
C13–G22–G46	C46	1.6[c]	0.22
	U13–A22	2.9	0.40
	U13–A22–A46	3.2	0.44
A9–A23–U12	U9	1.3[c]	0.18
	G9–C23–G12	5.9	0.82
	G9–G23–C12	5.1	0.71

[a] Reaction conditions as in Table I. ND is not detected. [b] Relative to wild-type tRNA^{Phe} transcript $k_{obs} = 7.2 \times 10^{-4}$ s^{-1}. [c] Cleavage at C48 seen as well, see Table III.

Mutations in Other Tertiary Interactions. From the data presented thus far, it appears that the lead cleavage rate is not only sensitive to changes in nucleotides that are coordinated to lead directly but can also be affected by nucleotide changes that disrupt the overall folding of the tRNA. To determine the sensitivity of the lead cleavage reaction to mutations that should disrupt the structure further away from Pb(1), a number of other mutations in tertiary interactions were assayed (Table II).

The two tertiary pairs U8–A14 and U54–A58 are both about 9 Å away from Pb(1) and have the same reverse Hoogstein pairing configuration. They are located in different regions of the tRNA structure and have a very different relationship to the Pb(1) binding site. The conserved U8–A14 pair stabilizes the P10 turn and lies within the central stack of tertiary interactions, insulated from Pb(1) by several stacking interactions. The conserved U54–A58 pair forms across the T loop and, by stacking between G53–C61 and G18–U55, maintains the lead-coordinating residues U59 and C60 in a looped out configuration (Figure 4). For both the U8–A14 and U54–A58 pairs, U→C, U→A, and A→G mutations were chosen since they were not expected to greatly disrupt the tertiary base pairing. Model building suggests that the U→C and A→G mutants can still form one of the two hydrogen bonds (Sampson et al., 1990), and as seen in the crystal structure of yeast initiator tRNA$_f^{Met}$ (Sussman & Podjarny, 1983), the U54A mutation can form an A54–A58 pair with only minimal distortion of the backbone. Despite the conservative nature of the mutations, all six mutations show a 4–14-fold decrease in the rate of lead cleavage. This result is less surprising for the U54 and A58 mutants since it is clear that the U54–A58 pair is important for positioning the U59–C60 loop. However, it is surprising that equally large effects on the cleavage rate are observed when the more distant U8–A14 interaction is mutated.

Inverting the highly conserved G53–C61 pair at the end of the T stem to C53–G61 results in a 9-fold decrease in the rate of lead cleavage (Table II). Romby et al. (1987) also noted that C61U resulted in a change in the T-loop conformation as judged by the ethylnitrosourea reactivity of P60 and suggested that it resulted from the loss of the hydrogen bond between N4 of C61 and P60. Since the C53–G61 mutation

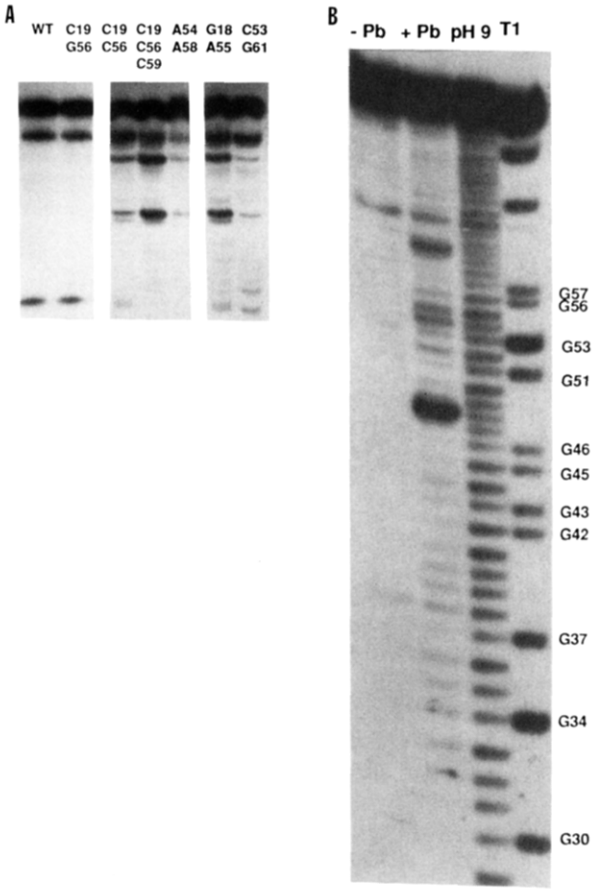

FIGURE 5: Cryptic cleavage site in certain tRNA mutants. (A) Cleavage of $tRNA^{Phe}$ and several mutants showing simultaneous cleavage at D17 and an additional site. (B) Location of the cryptic cleavage site for the 5′ ^{32}P labeled C19 mutant. Lanes as in Figure 1A.

would also disrupt this hydrogen bond and thereby destabilize the backbone in the U59–C60 loop, it is not surprising that the lead cleavage rate decreases substantially.

The strong effect on lead cleavage by conservative mutations in the rather distant U8–A14 pair prompted investigation of mutations of the two adjacent tertiary interactions that lie even further away from Pb(1). These are the C13–G22–G46 and A9–A23–U12 base triples. When G46 is mutated to a C, the two hydrogen bonds that connect the variable loop residue to the D-stem base pair are disrupted and the rate of lead cleavage is reduced 5-fold. In contrast, when C13–G22–G46 is changed to U13–A22–G46 or U13–A22–A46, the effect on lead cleavage is much less. Model building suggests that these two alternate base triples would maintain at least one hydrogen bond (Sampson et al., 1990). Similar but smaller effects are seen with the more distant A9–A23–U12 interaction. When A9–A23–U12 is changed to the G9–C23–G12 triple found in many other tRNAs, little or no effect on the lead cleavage rate is observed. However, if the tertiary interaction is disrupted by making A9U, the lead cleavage rate is reduced 2.5-fold. It is therefore apparent that mutations in $tRNA^{Phe}$ that disrupt the structure quite far from the Pb(1) site still affect the rate of cleavage.

A Cryptic Cleavage Site. For several of the mutations in Tables I and II, the reduced cleavage at U17 is accompanied by a substantial increase of cleavage at a new "cryptic" site. As shown in Figure 5A, the new cleavage products appear to be the same for every mutant. By use of the C56G mutant, this new cleavage site was shown to be after C48 in the variable loop (Figure 5B). The rates of cleavage at C48 for several transcripts are given in Table III. Although C48 cleavage is considerably slower than the normal cleavage rate at U17, some of the mutants cleave more rapidly at C48 than U17. Cleavage at C48 is not simply the result of a reduced rate of cleavage at U17 since a number of mutations reduce cleavage at U17 without showing detectable cleavage at C48 (Tables I and III).

Table III: Cleavage Rates of Mutant Yeast $tRNA^{Phe}$ Transcripts at C_{48}

mutation	k_{obs} ($s^{-1} \times 10^4$)	mutation	k_{obs} ($s^{-1} \times 10^4$)
C55	0.33	A54	0.35
A55	0.36	G58	0.29
C19	0.18	U9	0.45
C19–C59	0.51	C53–G61	0.33
G56	0.22		

Table IV: Cleavage of tRNA Transcripts by Lead[a]

transcript[b]	k_{obs} ($s^{-1} \times 10^4$)	rel k_{obs}
yeast $tRNA^{Phe}$	7.2	(1.0)
E. coli $tRNA^{Phe}$	0.4	0.06
E. coli $tRNA^{Phe}$ (U60C)	5.2	0.72
yeast $tRNA^{Arg \rightarrow Phe}$	12.0	1.67
B. subtilis $tRNA^{Phe}$	0.4	0.06
yeast $tRNA^{Met \rightarrow Phe}$	5.8	0.81

[a] Reaction conditions as in Table I. [b] See Figure 6 for sequences.

Many of the mutations that display cleavage at C48 are likely to alter the tertiary structure of the tRNA. For example, both the G19C and C56G mutations show the cryptic cleavage site, while the double mutant G19C, C56G that restores the tertiary base pair does not. Even the relatively subtle inversion of the G53–C61 base pair to C53–G61 results in enhanced cleavage at C48. The A9U and G46C mutations that disrupt tertiary interactions involving the D stem also show cleavage at the cryptic site. However, not all mutations that are believed to disrupt tertiary structure show cleavage at C48. For example, all of the mutations of the U8–A14 and G15–C48 pairs show a reduced rate of cleavage at U17 without enhancing C48 cleavage. The situation is even more complex for the G15–U48 and U54–A58 interactions where some of the mutations show cleavage at C48 while others do not, with no correlation with their ability to cleave at U17. Unfortunately, no clear conclusion can be drawn about the general features of mutations that produce enhanced cleavage at C48.

Sine the 2′ hydroxyl of ribose 48 is quite far away from any of the three known tightly bound lead atoms, it is unclear whether any of them is responsible for the new cleavage reaction. To test whether Pb(1) could cause cleavage at C48, the double mutant G19C, U59C was constructed where G19C was included to enhance cleavage of C48 and U59C was included to weaken binding of lead to Pb(1). As seen in Table III, this double mutant showed a normal rate of cleavage at C48, suggesting that Pb(1) is not responsible for the reaction.

Introducing a Lead Cleavage Site. Escherichia coli $tRNA^{Phe}$ has the same number of nucleotides as yeast $tRNA^{Phe}$ but differs in nucleotide sequence at 28 positions (Figure 6). It is likely that the two tRNAs have a very similar structure since an *E. coli* $tRNA^{Phe}$ transcript mutated from a U to a G at position 20 becomes an excellent substrate for yeast phenylalanyl synthetase (Sampson et al., 1989). Under standard conditions, the *E. coli* $tRNA^{Phe}$ transcript cleaves very slowly with lead, although some cleavage at U17 is observed (Table IV). This result was expected since *E. coli* $tRNA^{Phe}$ has a U at position 60 and the C60U mutation of the yeast $tRNA^{Phe}$ transcript showed a similar slow rate of cleavage (Table I). To introduce a lead cleavage site into *E.*

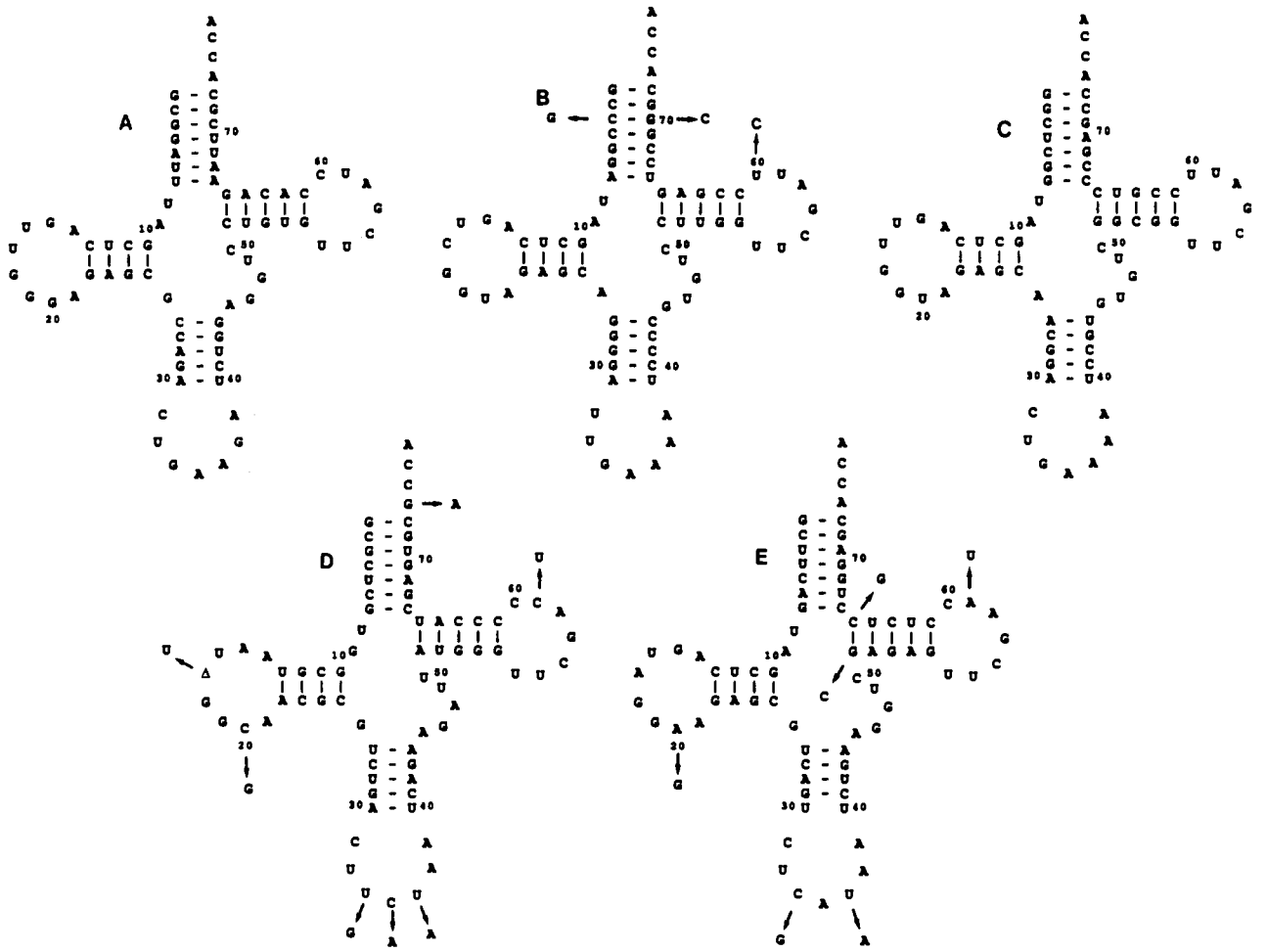

FIGURE 6: Five tRNA transcripts. (A) Yeast tRNA^{Phe}; (B) *E. coli* tRNA^{Phe} with the U60C mutation; (C) *B. subtilis* tRNA^{Phe}; (D) yeast tRNA^{Arg} with mutations making tRNA$^{Arg\rightarrow Phe}$; (E) yeast tRNA^{Met} with mutations making tRNA$^{Met\rightarrow Phe}$.

coli tRNA^{Phe}, we prepared the U60C mutation which should allow a lead ion to bind in an analogous position in this tRNA. As shown in Table IV, this mutant *E. coli* tRNA^{Phe} transcript cleaves at nearly the same rate as the yeast tRNA^{Phe} transcript. Thus, the remaining 27 differences between the two tRNA sequences, which are mostly in base-paired residues, do not affect the reaction.

Werner et al. (1976) have shown that native tRNA$_3^{Arg}$ cleaves quite poorly with lead at D16. This is likely to be the consequence of a C at position 59 and possibly because it lacks a residue at position 17. As part of our studies of the recognition at yeast tRNA^{Phe} by its synthetase (Sampson et al., 1989) we prepared tRNA$^{Arg\rightarrow Phe}$ which, among other changes, makes C59U and introduces a U residue at position 17 (Figure 6). As predicted, tRNA$^{Arg\rightarrow Phe}$ is now active in the lead cleavage reaction (Table IV).

Two other tRNA transcripts were tested for lead cleavage (Figure 6). Although *Bacillus subtilis* tRNA^{Phe} is likely to have a structure similar to that of yeast tRNA, it cleaves poorly with lead presumably because of the presence of a U60. Finally, tRNA$^{Met\rightarrow Phe}$ is a derivative of yeast tRNA^{Met} with six nucleotide changes including the crucial A59U (Sampson et al., 1989). As expected, this tRNA transcript cleaves rapidly with lead (Table IV).

Discussion

The specific cleavage of the tRNA^{Phe} transcript by lead at U17 was anticipated on the basis of the molecule's similarity to its fully modified counterpart. However, the transcript cleaves 2-fold more slowly than the native tRNA^{Phe} under a variety of conditions. The transcript also has a lower T_m than native tRNA^{Phe}, suggesting that it has a less ordered structure (Sampson & Uhlenbeck, 1988). The region of the D loop near the cleavage site shows a large temperature factor in the crystal (Westhof & Sundaralingam, 1986), indicating conformational flexibility in this part of the molecule. Since cleavage will only occur when the 2′ hydroxyl of ribose 17 in the flexible D loop is precisely positioned with respect to the bound lead, the increased flexibility of the unmodified transcript would be expected to lower the cleavage rate.

Of the 14 modified nucleotides in tRNA^{Phe}, 5 (T54, m^{1}A58, Ψ55, D16, and D17) are located near Pb(1). Since D16 and D17 are pointed away from the bound lead and these positions can be mutated to C without altering the cleavage rate in the transcript, it is unlikely that these modifications play a role in lead cleavage. The absence of the remaining three modifications in the transcript are most likely to be responsible for the more flexible structure and the resulting slower lead cleavage rate. The T54–m^{1}A58 pair is involved in maintaining the T-loop structure and helps to position the lead-coordinating residues U59 and C60. Ψ55 is paired with G18, thereby holding the substrate phosphodiester backbone close to the active lead, and also stabilizes the T-loop structure. Since even quite conservative mutations in these positions reduce lead cleavage, it is clear that they contribute substantially to the structure in the region. Both T and Ψ stack on neighboring nucleotides more effectively than U and form more stable Watson–Crick pairs. Thus, it seems likely that these modifications increase the stability of the tertiary pairs as well, thereby stabilizing the whole tRNA structure. The rT54

modification was clearly demonstrated to be responsible for increasing the stability of *E. coli* $tRNA^{Met}$ (Davenloo et al., 1979).

Conservative mutations of the two pyrimidines that coordinate the lead substantially reduced the lead cleavage rate. The changes must alter the manner in which the lead atom is coordinated. Thus, when U59 is changed to C59, the carbonyl oxygen at 4 [2.2 Å from Pb(1)] is changed to an amino group and the imino proton at 3 [3.6 Å from Pb(1)] is lost. Similarly, the C60U mutation changes the N3 [2.8 Å from Pb(1)] to a NH and the NH3 at 4 [3.5 Å from Pb(1)] to a carbonyl oxygen. It is unlikely that these changes could have a large effect on the affinity of lead. Although in model compounds lead binds preferentially to carbonyl oxygen over an aromatic nitrogen (Yu & Fritchie, 1975), lead shows little preference between nitrogen and oxygen at the two other sites in tRNA (Brown et al., 1985). Thus, it is more likely that the lead remains bound to the mutant tRNAs but is positioned in such a way that cleavage does not occur.

Mutations which disrupt the tertiary interactions that maintain the structure of the T loop and hold the D loop to the T loop alter the lead cleavage rate. Subtle changes that only disrupt a single hydrogen bond (U54C, A58G and G53C, C61G) or slightly alter the backbone (U54A) can substantially reduce the cleavage rate. These results are not surprising considering the fact that these tertiary interactions are important both in positioning the lead-coordinating nucleotides U59 and C60 and in keeping the substrate 2′ hydroxyl near the lead. The sensitivity of this region of the tRNA structure to mutation has been encountered by others (Reilly & RajBhandary, 1986; Romby et al., 1987).

It is striking that mutations which disrupt tertiary interactions quite far from Pb(1) still reduce the rate of cleavage. Presumably, the structural alteration caused by the mutation is propagated through the stacked nucleotides to alter the structure near Pb(1). Krzyzosiak et al. (1988) have shown that the removal of the anticodon nucleotide Y37 from native yeast $tRNA^{Phe}$ by acid treatment increases the rate of lead cleavage. Another example of such a long-range structural distortion is the disruption of the G19–C56 pair in the yeast $tRNA^{Asp}$ crystal lattice that appears to be the result of an anticodon–anticodon interaction (Moras et al., 1986). The sensitivity of lead cleavage to mutations over a large region of tRNA makes it an excellent independent assay of tRNA structure in structure–function studies (J. R. Sampson, A. B. DiRenzo, L. S. Behlen, and O. C. Uhlenbeck, unpublished results).

Interestingly, several mutations that disrupt tertiary interactions not only reduce the rate of cleavage at U17 but greatly increase the rate of cleavage at C48. The mechanism of this cryptic cleavage reaction is unclear, but presumbly involves a bound lead in close proximity to ribose 48. It is unlikely that one of the three known lead binding sites is involved. All three are quite far away from ribose 48, and when Pb(1) is disrupted by mutation, the cryptic cleavage can still be observed. Since the cleavage at C48 is quite slow, it may be the result of a lead bound more weakly at another site. The magnesium binding site Mg(1), which is coordinated to phosphates 8 and 9 about 7 Å away from the 2′ hydroxyl of ribose 48, is a possible candidate. Since little cleavage is observed with the native sequence, it is necessary to hypothesize that the mutations in the tertiary structure alter the conformation of the transcript sufficiently to bring ribose 48 into close proximity to the lead.

The data on mutant $tRNA^{Phe}$ transcripts make it possible to interpret the presence or absence of lead cleavage in the D loop for a variety of other tRNAs. The observation that yeast $tRNA^{Val}$ cleaves rapidly while yeast $tRNA^{Asp}$, $tRNA^{Tyr}$, $tRNA^{Trp}$, $tRNA_3^{Arg}$, and *E. coli* $tRNA_f^{Met}$ cleave poorly (Werner et al., 1976) can be understood by the fact that only $tRNA^{Val}$ contains the U59–C60 sequence necessary to properly bind and position the lead. The *E. coli* and *B. subtilis* $tRNA^{Phe}$ transcripts that have U59–U60 also cleave poorly. We have confirmed the importance of the U59–C60 sequence for cleavage by demonstrating that both the U60C *E. coli* $tRNA^{Phe}$ and the $tRNA^{Arg\rightarrow Phe}$ transcripts cleaved rapidly with lead. However, it appears that other sequences at positions 59 and 60 can coordinate lead for cleavage since yeast $tRNA_2^{Arg}$ cleaves rapidly at D16 and contains G59–U60 (Werner et al., 1976). The sequence requirements for nucleotides around the cleavage site have also been partially established. It is clear that many nucleotides can be accommodated at positions 16 (D, U, and C) and 17 (D, U, A, and C) without affecting cleavage. Since yeast $tRNA^{Val}$ contains an extra nucleotide in the D loop (D20a) and still cleaves at position 17, some size flexibility in this loop is also permitted. Additional mutagenesis experiments will be necessary to fully establish what is required for lead cleavage.

The structural requirements for the cleavage of $tRNA^{Phe}$ by lead show several interesting similarities to the structural requirements of the hammerhead self-cleaving domain suggested by a comparison of eight natural examples (Hutchins et al., 1986; Buzayan et al., 1986b; Forster et al., 1987; Epstein & Gall, 1987). Since the lead cleavage reaction occurs in a known RNA structure, the comparison provides insight into how the hammerhead domain might function. In both cases, the cleavage domain consists of RNA helices (four for tRNA, three for the hammerhead) and a number of conserved or semiconserved nucleotides (10 or more for tRNA, 13 for the hammerhead) that are primarily single stranded in the secondary structure. Since in both cases most of the helical base pairs can vary in sequence, it is clear that the helical regions are primarily needed to maintain the structure of the domain and not for the catalytic activity. On the basis of the data presented here, only two single-stranded nucleotides in lead cleavage domain are involved directly in the mechanism by coordinating the active lead ion. The remaining residues are needed for the proper folding of the domain since often only unique combinations of bases can provide the correct tertiary interaction. Although the folded hammerhead domain is likely to involve different tertiary interactions, a similar situation may exist where most of the conserved nucleotides are essential for the folding of the molecule. Finally, it is interesting to note that for both cleavage reactions the nucleotide 5′ to the cleavage site can vary in sequence. This is easy to understand in the case of the tRNA since U17 is pointed outward, allowing the ribose to be in contact with the reactive lead. Perhaps a similar situation exists in the hammerhead domain.

Registry No. Pb, 7439-92-1; U, 66-22-8; C, 71-30-7.

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Role of the Tertiary Nucleotides in the Interaction of Yeast Phenylalanine tRNA with Its Cognate Synthetase†

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ABSTRACT: In vitro transcription by T7 RNA polymerase was used to prepare 32 different mutations in the 21 nucleotides that participate in the 9 tertiary base pairs or triples of yeast $tRNA^{Phe}$. The mutations were designed either to disrupt the tertiary interaction or to change the sequence without disrupting the structure by transplanting tertiary interactions present in other tRNAs. Steady-state aminoacylation kinetics with purified yeast phenylalanyl synthetase revealed little change in reaction rate as long as a tertiary interaction was maintained. This suggests that the tertiary nucleotides only contribute to the folding of $tRNA^{Phe}$ and do not participate directly in sequence-specific interaction with the synthetase.

The crystal structure of yeast $tRNA^{Phe}$ contains nine tertiary interactions that contribute to the folded structure of the tRNA (Figure 1) (Robertus et al., 1974; Kim et al., 1974). With one exception, these tertiary interactions involve nonstandard base pairs and in three cases involve interaction between three nucleotides (Figure 2). A comparison of more than 200 available nonmitochondrial tRNA sequences reveals that of the 21 nucleotides involved in these interactions 9 are completely conserved and 9 more are semiconserved as either a purine or pyrimidine (Grosjean et al., 1982). The observed covariance of the semiconserved nucleotides in other tRNA sequences suggested that many tertiary interactions can be replaced by other nucleotides with only minimal alterations of the phosphodiester backbone (Robertus et al., 1974; Klug et al., 1974; Kim, 1976). The crystal structures of yeast $tRNA^{Asp}$, yeast $tRNA^{Metf}$, and *Escherichia coli* $tRNA^{Metf}$ confirmed several of these alternate tertiary motifs (Westhof et al., 1985; Schevitz et al., 1979; Woo et al., 1980). In addition to their structural role, it has been proposed that several of the tertiary nucleotides are important for recognition by various enzymes that interact with tRNA. For example, the conserved TΨC sequence has been proposed to interact with the ribosome (Erdmann et al., 1973), and a subset of the conserved nucleotides in yeast $tRNA^{Phe}$ and $tRNA^{Leu}$ precursors may be specifically recognized by the tRNA splicing apparatus (Reyes & Abelson, 1988; Mattoccia et al., 1988).

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