Cleavages Induced by Different Metal Ions in Yeast tRNA^{Phe} U59C60 Mutants†

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ABSTRACT: The U59 and C60 residues, which form the strong Pb(II) ion binding site in yeast tRNA^{Phe}, were systematically mutated, and the effects of individual mutations on cleavages induced by various metal ions were analyzed. It turned out that the presence of C60 is essential for efficient cleavage of the D-loop by Pb(II), Eu(III), and Mg(II) ions. On the other hand, manganese ions were capable of cleaving these mutants with an efficiency similar to that observed for the wild type transcript. Moreover, in all C60 mutants, the main Mn(II) cleavage site was shifted by one phosphate from P16 to P17. Mutations of U59 did not affect so dramatically the efficiency and specificity of the D-loop hydrolysis induced by all studied metal ions. In the G59C60 mutant cleaved by Pb(II) ions, new cuts took place in the T-stem at P63-P65. Also, the C60 mutants were cleaved more strongly at P49 by Pb(II) ions. In G59C60 and A59C60 as well as in all C60 mutants, the Mg(II) and Mn(II) cleavage at P61 was suppressed. Nevertheless, the changes in overall tRNA structure resulting from U59 and C60 mutations were rather subtle. The studied mutants showed S_1 and V_1 nuclease digestion patterns practically indistinguishable from those observed in the wild type transcript. The metal ions are shown to be well-suited for monitoring the local changes in the structure of the investigated tRNA variants and when used as a set of probes can give a more complete picture of changes that occur in transcripts as a result of a mutation.

It has been known for a long time that some metal ions like Pb(II), Co(II), Ni(II), Cu(II), Zn(II), Mn(II), and Mg(II) depolymerize RNA homopolymers such as poly(A), poly(U), poly(C), and poly(I) (Butzow & Eichorn, 1965; Farkas, 1968). The highest rates of cleavages were observed with Pb(II) followed by Zn(II) ions (Farkas, 1968). Later studies have shown that Pb(II) ions were also capable of cleaving tRNA molecules with high efficiency (Werner et al., 1976). The detailed mechanism of specific tRNA scission by metal ions comes from crystallographic studies and describes Pb(II) ion-induced hydrolysis of yeast tRNA^{Phe} (Brown et al., 1983, 1985; Rubin et al., 1983). The Pb(II) ion designated as Pb(1), which causes the tRNA cleavage, is placed in a pocket formed by nucleotides of the T- and D-loops, and it is bound directly to the C60 as well as U59 residues. At a pH close to the pK_a value, the lead hydrate exists in a partially deprotonated form and subtracts a proton from the ribose 2′-OH group of D17. The subsequent steps of tRNA cleavage involve the attack of the 2′-O$^-$ nucleophile on the phosphorus atom, the formation of a pentacoordinate phosphate intermediate, and finally the scission of the phosphodiester bond giving 2′,3′-cyclic phosphate and 5′-OH termini. On the basis of this mechanism also, the specific tRNA cleavages induced by Pb(II), Eu(III), Mg(II), and Mn(II) ions in solution could be explained (Krzyżosiak et al., 1988; Deng & Termini, 1992; Pan et al., 1993; Ciesiołka et al., 1989a; Marciniec et al., 1989a; Wrzesinski et al., 1995). The Pb(II), Eu(III), Mg(II), and Mn(II) ions are known to have different preferences for nucleic acid ligands. The "hard" metal ions Mg(II) and Eu(III) tend to bind preferentialy to the oxygen ligands and phosphates, while the "soft" ions Pb(II) and Mn(II) interact rather with the aromatic nitrogen atoms of nucleic acid bases (Saenger, 1984; Pan et al., 1993). The available data on different binding preferences of these four metal ions to RNA structures are, however, fragmentary, and they come mainly from the analysis of several "tight" metal ion binding sites in yeast tRNA^{Phe} crystals.

It has been postulated that the specific and highly efficient cleavage of the D-loop by Pb(II) ions depends on the presence of U59 and C60 residues in the T-loop (Brown et al., 1983, 1985). The analysis of cleavages induced in several native tRNA molecules has led us to similar conclusions (Krzyżosiak et al., 1988). Further analysis of natural tRNAs suggested, however, that U59 may not be as important as C60 in tRNA cleavage (Ciesiołka et al., 1989b). The importance of U59 in this reaction was proven later by showing the reduced susceptibility of the yeast tRNA^{Phe} C59C60 mutant to lead(II) hydrolysis (Behlen et al., 1990). It was also demonstrated in a very elegant and convincing experiment that *Escherichia coli* tRNA^{Phe}, which contains the U59U60 sequence and is resistant to lead cleavage, became highly reactive when uracil at position 60 was replaced by cytosine (Behlen et al., 1990).

The purpose of this work was to obtain more information on structure requirements for specific hydrolysis of yeast tRNA^{Phe} by various metal ions. We focused more closely on the role played by U59 and C60 in formation of the metal ion binding pocket. We wanted to find out how other bases influence the Pb(II), Mg(II), Mn(II), and Eu(II) binding and subsequent cleavages, when present at these positions. This study extends the earlier mutagenic analysis of the same metal ion binding site performed by Uhlenbeck's group (Behlen et al., 1990). It contributes to a better understanding of the metalloribozyme system considered a model for certain types of catalytic RNAs.

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MATERIALS AND METHODS

Materials. Materials used in this study were from the following sources. [γ-^{32}P]ATP (5000 Ci/mmol) was from Amersham, and all chemicals were from Serva. Polynucleotide kinase and T7 RNA polymerase were purchased from New England Biolabs. Nucleases S_1, V_1, and T_1 were from Pharmacia.

Preparation of Yeast tRNA^{Phe} Mutants. All mutants were prepared by PCR primer-directed mutagenesis of the synthetic yeast tRNA^{Phe} gene which was cloned in the pUC19 vector as described previously (D. Michalowski, unpublished data). The recombinant plasmid DNA was prepared by the standard method (Sambrook et al., 1989) and purified on a cesium chloride gradient. The PCR F primer was complementary to nucleotides −19 to +13 of the yeast tRNA^{Phe} gene flanked by T7 RNA polymerase promoter. The mutagenic R primers were complementary to nucleotides 56−76 except for mutations at position T59 or C60. All PCR primers were synthesized on an Applied Biosystems model 392 DNA/RNA synthesizer, deproctected, and purified on OPC columns according to the manufacturer's recommendation. The PCR was done on a Perkin-Elmer 480 DNA thermal cycler, and the reaction mixture (20 μL) contained 10 mM Tris-HCl (pH 8.3), 15 mM $MgCl_2$, 50 mM KCl, a 200 μM concentration of each dNTP, 5 ng of DNA template, 26.5 pmol of each primer, and 1.25 u of Taq polymerase (Perkin-Elmer/Cetus). Amplifications were done in 30 cycles: 1 s at 94 °C, 1 s at 55 °C, and 1 s at 72 °C following the protocol for fast and economical PCR (Sobczak et al., 1995). The PCR products were extracted with phenol and precipitated with ethanol, and DNA was finally dissolved in H_2O and used for transcription *in vitro* without further purification. The identity of each mutant was confirmed by nucleotide sequencing.

In Vitro Transcription. The transcription reaction mixture contained 20 mM Hepes (pH 7.5), 100 mM NaCl, 20 mM $MgCl_2$, 2 mM spermidine, 1.0 mM NTPs, 3.0 mM guanosine, 500 u/mL T7 RNA polymerase, and the DNA template at a 1 μM concentration. After 4 h of incubation at 37 °C, the mixture was phenol extracted and ethanol precipitated. The *in vitro* transcripts were purified on a denaturing 12.5% polyacrylamide gel, and then the RNA band corresponding to full size tRNA was identified under UV light, excised, and eluted from the gel with 0.3 M potassium acetate at pH 5.1, 1 mM EDTA, and 0.1% SDS. The transcript was then ethanol precipitated, dissolved in water, and stored at −20 °C before use.

Labeling of tRNA. The wild type *in vitro* transcript and mutants of yeast tRNA^{Phe} were phosphorylated with [γ-^{32}P]-ATP and polynucleotide kinase. The labeled RNAs were purified by electrophoresis and identified in the gel by autoradiography, and tRNA was recovered as described above.

Metal Ion-Induced Cleavage. Prior to reaction, the labeled transcripts were supplemented with the corresponding unlabeled RNA carrier to obtain a final tRNA concentration of 8 μM and subjected to the standard denaturation/renaturation procedure (Krzyżosiak et al., 1988). The reactions were performed in 40 mM NaCl and 10 mM Tris-HCl buffer of the appropriate pH. The lead and europium reactions were carried out in the presence of 10 mM magnesium chloride. This Mg(II) concentration which was found to be optimum for the efficient Pb(II)-induced cleavage of the wild type transcript (Behlen et al., 1990) was also used in the reactions with all U59C60 mutants. As these studies were not designed to obtain the quantitative kinetic parameters, we did not attempt to determine the relative binding constants of Pb(II), Eu(II), and Mg(II) ions to analyzed tRNA variants. The details of reaction conditions are specified in the figure legends. All reactions were stopped by mixing their aliquots with 8 M urea/dyes containing 20 mM EDTA and loaded on a 15% polyacrylamide, 0.75% bis-acrylamide, and 7 M urea gel. Electrophoresis was at 1500 V for 3 h, followed by autoradiography at −80 °C with an intensifying screen.

Nuclease Mapping. Labeled transcripts were supplemented with homologous unlabeled RNAs as described above. Limited digestions with nucleases S_1 and V_1 were carried out in a reaction mixture containing 10 mM Tris-HCl (pH 7.2), 40 mM NaCl, and 10 mM $MgCl_2$. In case of S_1 digestion, $ZnCl_2$ was also present at 1 mM. Reactions were performed at 37 °C for different time intervals with a 300 u/mL concentration of nuclease S_1 and a 150 u/mL concentration of nuclease V_1. The reactions were stopped by adding 8 M urea/20 mM EDTA and analyzed by polyacrylamide gel electrophoresis.

Analysis of Reaction Products. In order to assign the cleavage sites, the products of metal ion-induced hydrolysis or nuclease digestion were run along with the products of alkaline RNA hydrolysis and limited nuclease T_1 digestion of the same tRNA. The alkaline hydrolysis ladder was generated by incubation of tRNA solution together with $^1/_5$ volume of formamide in boiling water for 10 min. Partial nuclease T_1 digestion of tRNA was performed in denaturing conditions [50 mM sodium citrate (pH 4.5) and 7 M urea] with 0.1 unit of the enzyme. The reaction mixture was incubated for 10 min at 50 °C.

RESULTS

Metal Ion-Induced Cleavages in the Wild Type Transcript. In the experiments described in this paper, we analyzed cleavages induced in all U59 and C60 mutants and compared them with those induced in a reference molecule which was the wild type transcript (Figure 1). Previous experiments have shown that Pb(II)-, Mg(II)-, Eu(III)-, and Mn(II)-induced hydrolysis patterns in the native yeast tRNA^{Phe} molecule and in its corresponding *in vitro* transcript were similar (D. Michałowski, unpublished data), except for some quantitative differences specified below. First, the decreased efficiency of the main cleavage in the D-loop was found in the transcript molecule. This effect was observed for all studied metal ions, perhaps except for europium ions where this tendency was less clear. In the case of Pb(II) ions, the cleavages at P17 and P16 were also suppressed. Likewise, the cuts induced by Mg(II) and Mn(II) ions at P20 were of lower intensity in the transcript. On the other hand, stronger hydrolysis of the transcript by Mg(II) and Mn(II) ions was observed at P17 and P18. New Eu(III) cleavage sites appeared in the transcript molecule at P20 and P21, and new Mg(II) and Mn(II) cuts were found at P21 and P61. Moreover, the *in vitro* transcript was susceptible to weak cleavages in the entire anticodon loop.

Pb(II)-Induced Hydrolysis in tRNA^{Phe} Mutants. The efficiency of hydrolysis at the main cleavage site at P18,

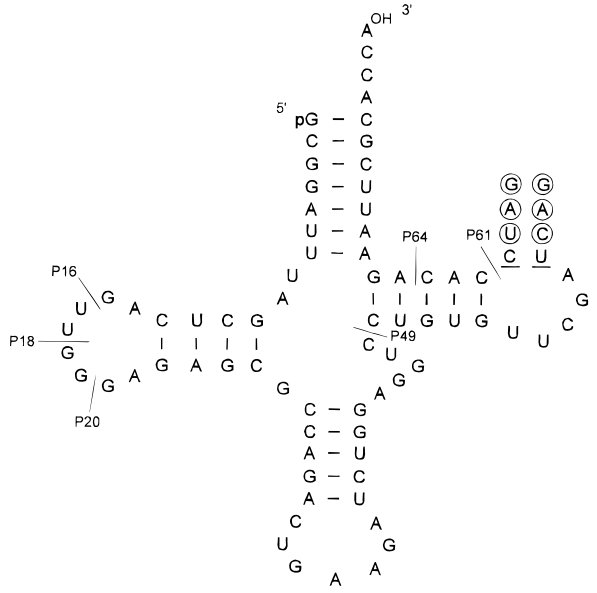

FIGURE 1: Secondary structure of the yeast tRNA^{Phe} transcript. The mutated bases U59C60 are underlined, and bases introduced in these positions are circled. Positions of metal ion-induced cleavages discussed in the text are marked.

observed in the wild type transcript, is retained in G59C60 and A59C60 mutants and is reduced in the C59C60 mutant (Figure 2A). The cleavage of the D-loop is more specific in A59C60 and C59C60 mutants in which the cuts at P16 and P17 are suppressed. Also in the G59C60 mutant, the cut at P17 is weaker while the cleavage at P16 is stronger. Surprisingly, in the G59C60 mutant, new cleavage sites appear in the T-stem. There are three cleavages observed in that region when electrophoresis is performed for a longer time (data not shown). The main one is at P64, and the weaker cuts occur at P63 and P65. An entirely different cleavage pattern is observed in cytosine 60 mutants (Figure 3A). In U59G60 and U59A60 mutants, the Pb(II)-induced cleavage of the D-loop is completely inhibited. In the U59U60 mutant, the efficiency of cleavages at P17 and P18 is strongly reduced. The cut at P16 is also suppressed, although to a lower extent. Interestingly, the new cut at P49 in the variable region appears in all C60 mutants.

In the crystal structure of yeast tRNA^{Phe}, the Pb(1) ion that cleaves the D-loop is directly coordinated to the O4 oxygen atom of uracil 59 and N3 endocyclic nitrogen atom of cytosine 60 (Figure 6). Previous studies on Pb(II)-induced cleavages in some native tRNA molecules suggested the primary importance of C60 for efficient cleavage in the D-loop (Ciesiołka et al., 1989b). The studied methionine-specific tRNAs that contained adenine or guanine residues at position 59 and a cytosine residue at position 60 were cleaved in the D-loop with a high efficiency by Pb(II), Eu(III), and Mg(II) ions. Also in G59C60 and A59C60 mutants described in this paper, a highly efficient cleavage by Pb(II) is observed. Only in the case of the C59C60 mutant is the hydrolysis significantly reduced which is in good agreement with the results obtained earlier by other authors (Behlen et al., 1990). A very low intensity of cleavage at P18 was also observed in natural yeast tRNA^{Asp} and *E. coli* tRNA^{Phe} that contain the U59U60 sequence (Krzyżosiak et al., 1988; Marciniec et al., 1989b). The same was also the case for the yeast tRNA^{Phe} U59U60 mutant (Behlen et al., 1990).

Eu(III)-Induced Hydrolysis in tRNA Mutants. Mutation of the uracil residue at position 59 does not affect very much the Eu(III)-induced hydrolysis (Figure 2B). The efficiency of cleavage at P16 is very similar for each U59 mutant. Only in the case of purines present at that position is a significant reduction of the intensity of the cuts at P17 and P18 observed. Still higher reduction of cleavage efficiency and some differences in the specificity of the hydrolysis are observed in all three C60 mutants (Figure 3B). In the wild type molecule, three strong cuts appear in the D-loop at P16−P18 and cleavages at P19−P21 are very weak. In the case of mutants, all P16−P22 phosphodiester bonds are susceptible to cleavage at a high concentration of Eu(III) ions (Figure 2B, lanes D and E).

In the crystal structure of yeast tRNA^{Phe}, five strongly bound lanthanide ions were identified (Jack et al., 1977). Two of them coordinate to D- and T-loop ligands and occupy approximately the same binding sites as Mg(1) and Mg(3) ions (Figure 6). The similarity between the lanthanide and Mg(II) binding sites is also seen in cleavages induced by these ions in several tRNA variants (compare Figures 2B,C and 3B,C).

Mg(II)-Induced Hydrolysis in tRNA Mutants. Significantly higher efficiency of Mg(II)-induced cuts is observed in G59C60 and A59C60 mutants than in the wild type transcript (Figure 2C). Moreover, the hydrolysis is more specific as several cuts that appeared at P18, P20, and P21 in the D-loop of the wild type transcript are almost completely absent in these mutants. Also, the cleavage at P61 in the T-loop does not occur in G59C60 and A59C60 mutants. On the other hand, the hydrolysis induced by Mg(II) ions in the C59C60 mutant highly resembles that observed in the wild type molecule. More profound are differences observed in C60 mutants (Figure 3C). Hydrolysis of the U59G60 and U59A60 mutants in the D-loop is strongly suppressed. The cleavages at P16, P18, and P20 that take place in the wild type transcript are practically absent in these mutants, and the strongest of the generally weak cuts appears at P17. The cleavage pattern observed in the U59U60 mutant is, as in the case of the C59C60 mutant, very similar to that of the wild type transcript, but its hydrolysis requires a higher concentration of magnesium ions. Moreover, the cut at P61 which is present in the wild type transcript is absent in all mutants.

In the monoclinic as well as orthorhombic forms of the yeast tRNA^{Phe} crystal structure, several strongly bound magnesium ions were identified, two of them in the D-loop (Hingerty et al., 1978; Holbrook et al., 1977; Jack et al., 1977; Quigley et al., 1978). The ion designated as Mg(3) is coordinated in both crystal forms directly to phosphate G19 and via a water bridge to several other ligands, N7 G20, O6 G20, O4 U59, and N4 C60 among them. The Mg(2) ion is bound directly to phosphates adjoined to G20 and A21 residues (Figure 6). The strong requirement for a cytosine presence at position 60 for the efficient magnesium ion-induced hydrolysis suggests that the Mg(3) ion is probably involved in the D-loop cleavage. Only the Mg(3) ion is bound via a hydrated shell to U59C60 residues. The importance of cytosine 60 for efficient Mg(II)-induced cleavage of tRNA was suggested earlier, on the basis of the results obtained with yeast and *E. coli* tRNA^{Phe} (Marciniec et al., 1989b). The native *E. coli* tRNA^{Phe} that contains the

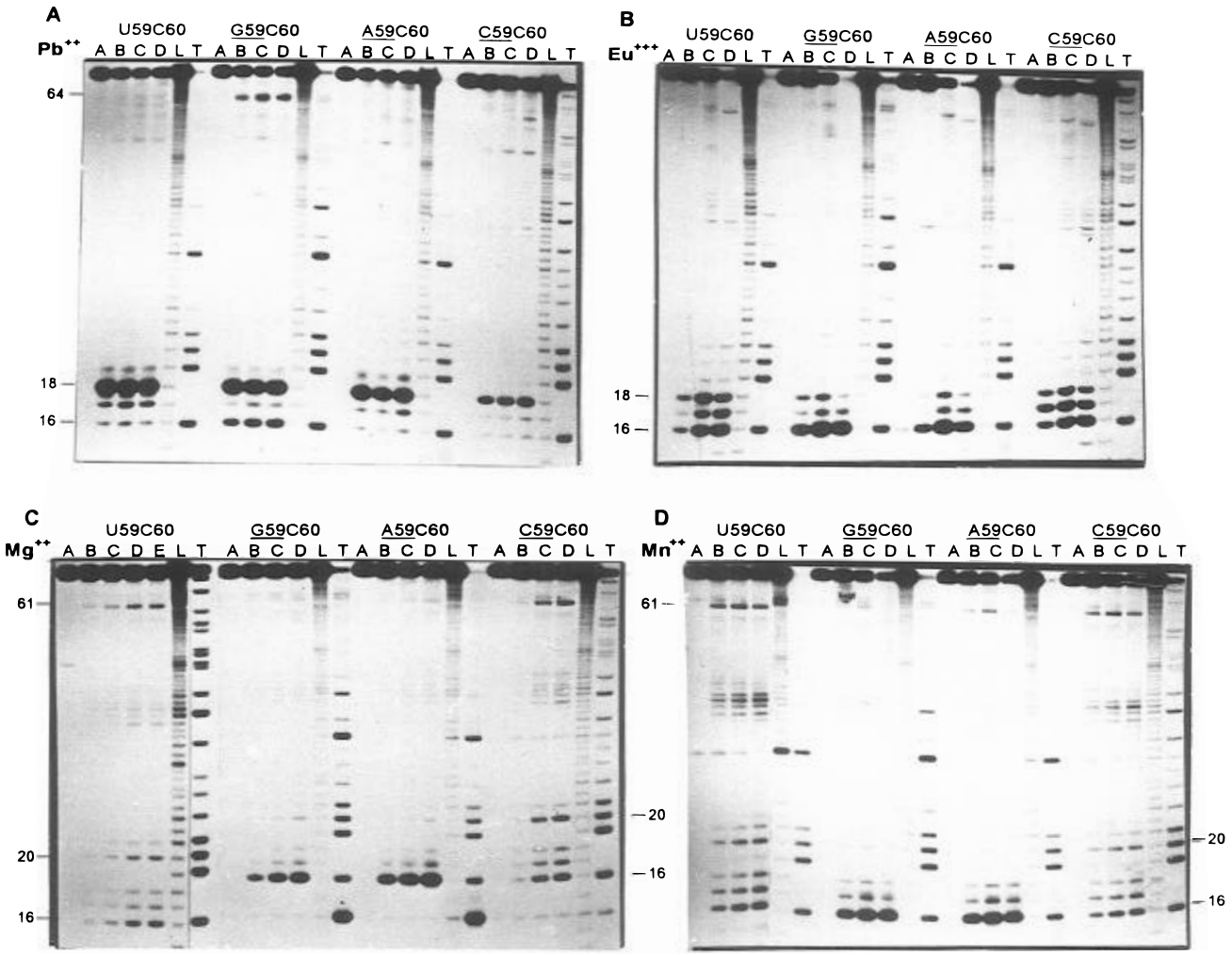

FIGURE 2: Specificity of hydrolysis of 5′-labeled yeast $tRNA^{Phe}$ wild type *in vitro* transcript and its U59 mutants induced by (A) Pb(II), (B) Eu(III), (C) Mg(II), and (D) Mn(II) ions. Reaction conditions: Pb(II), (lanes B−D) 0.5, 1, and 2.5 mM, respectively, pH 7.2, 20 min; Eu(III), (lanes B−D) 25, 50, and 100 μM, respectively, pH 7.2, 12 h; Mg(II) wild type transcript, (lanes B−E) 0.3, 1, 3, and 10 mM, respectively; Mg(II) mutants, (lanes B−D) 1, 3, and 10 mM, respectively, pH 8.5, 12 h; and Mn(II), (lanes B−D) 1, 3, and 10 mM, respectively, pH 8.0, 12 h. Lanes: A, reaction control; F, formamide ladder; and T, limited hydrolysis by RNase T_1.

U59U60 sequence was very poorly cleaved by magnesium ions.

Mn(II)-Induced Hydrolysis in tRNA Mutants. In the case of the wild type transcript and the U59 mutants, the hydrolysis pattern (Figure 2D) resembles that obtained with Mg(II) ions (Figure 2C). Interestingly, some enhancement of cleavage efficiency at P16 is observed in mutants containing purines in position 59. On the other hand, the cleavage at P61 in the T-loop is suppressed in these mutants, especially in G59C60. The Mn(II) hydrolysis pattern observed in the C59C60 mutant is practically identical to that of the wild type transcript, only the intensity of cuts in the latter molecule is somewhat higher. It should also be stressed that mutants containing purines at position 59 are cleaved in an almost identical way by Mn(II) and Eu(III) ions (compare panels D and B of Figure 2). This similarity is even higher than for Mn(II) and Mg(II) ions (Figure 2). It suggests that Eu(III) and Mn(II) binding sites in these mutants are identical and very similar to the Mg(II) binding site. These Eu(III), Mg(II), and Mn(II) binding sites are, however, significantly different from those present in the wild type transcript and in the C59C60 mutant (Figure 2C,D). More important differences in hydrolysis patterns are seen in C60 mutants (Figure 3D). First of all, the main cleavage site is shifted from P16 to P17, and this effect is most profound for U59G60 and U59A60 mutants. A similar shift of the main cleavage site from P18 to P17 was observed in the $tRNA^{Phe}$ U8G mutant cleaved by Pb(II), Eu(III), Mg(II), and Mn(II) ions (D. Michałowski, unpublished data) as well as in one of the $tRNA^{Phe}$ variants obtained by *in vitro* selection of tRNA motifs cleaved by Pb(II) ions (Pan & Uhlenbeck, 1992). Thus, not only bases present at position 60 but also base changes occurring elsewhere in the molecule can modify the structure of the metal ion binding pocket in a way such that the site of the major cut is shifted.

Nuclease Mapping. The S_1 and V_1 nucleases are widely used in RNA structure probing (Ehressman et al., 1989; Knapp, 1989). We have applied those probes along with metal ions in analysis of the tRNA structural changes induced by U59C60 mutation. As in the case of metal ions, the reference molecule was a well-characterized wild type transcript, the structure of which was probed with these nucleases earlier (D. Michałowski, unpublished data). In Figure 4, the digestion patterns of U59U60 and U59G60 mutants are compared with those obtained for the reference molecule. The results of nuclease digestion show that single-base changes at position 60 in the T-loop have practically no influence on the specificity of the cuts induced by S_1

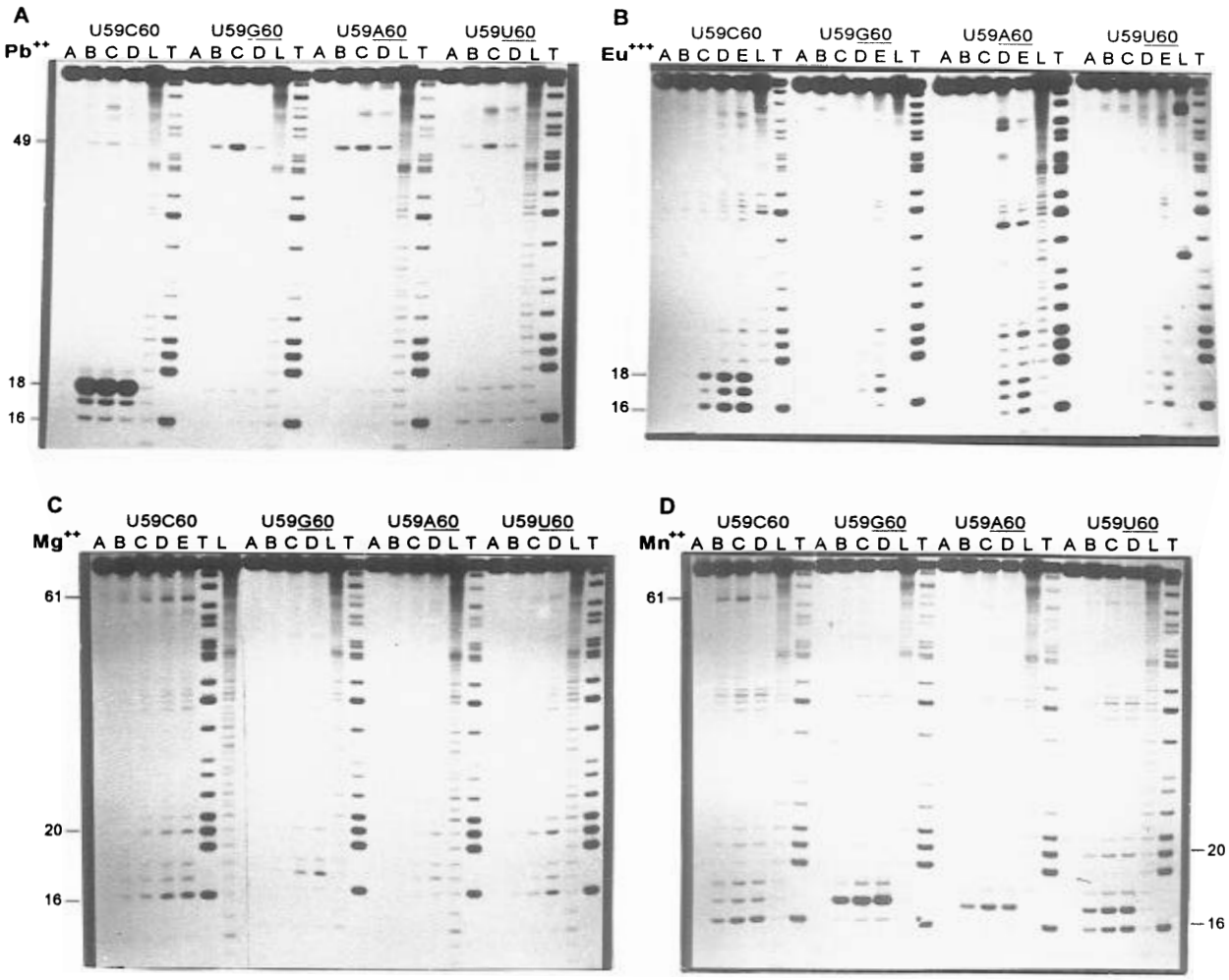

FIGURE 3: Specificity of hydrolysis of 5′-labeled yeast tRNA^{Phe} wild type *in vitro* transcript and its C60 mutants induced by (A) Pb(II), (B) Eu(III), (C) Mg(II), and (D) Mn(II) ions. The conditions of the reactions were identical to those described in the legend to Figure 2. Except in the case Eu(II), lanes B−E correspond to 10, 25, 50, and 100 μM concentrations, respectively.

nuclease in the anticodon loop and by V_1 nuclease in the D- and anticodon stem.

DISCUSSION

The involvement of U59C60 bases in formation of the Pb(II) ion binding site in yeast tRNA^{Phe} crystals is well-documented (Brown et al., 1983, 1985; Rubin & Sundaralingam, 1983). However, the necessity of both bases for the efficient cleavage of this tRNA by Pb(II) ions has been questioned, on the basis of results obtained with other natural tRNAs (Ciesiołka et al., 1989b). The data presented in this paper validate our earlier doubts and show that the presence of cytosine at position 60 is in fact more important for this very efficient and specific reaction to occur. The replacement of this cytosine residue by any other base almost completely inhibits the Pb(II)-induced cleavage. A similar effect, although less profound, is observed in Mg(II) and Eu(III) ion-induced hydrolysis of yeast tRNA^{Phe} C60 mutants deprived of base and sugar modifications. However, the reactions promoted by Mn(II) ions show distinct characteristics. The intensity of the main cleavage in the D-loop is in all C60 mutants similar to that observed in the wild type transcript. Moreover, the major cut is shifted by one nucleotide toward the 3′-end of the molecule. This shift is also observed in the weaker D-loop cuts induced by Mg(II) ions in U59C60 and U59A60 mutants (Figure 5).

The inspection of known sequences of elongator tRNAs shows that position 60 is most frequently occupied by uracil (73%), followed by cytosine (20%), by adenine (7%), and very rarely by guanine (Steinberg et al., 1993). According to our earlier observations, all analyzed native tRNAs containing a cytosine residue at position 60 were efficiently cleaved in the D-loop by Pb(II) ions. These included tRNA^{Phe}, tRNA^{Val}, and tRNA^{Met} from yeast and tRNA^{Phe} and tRNA^{Met} from lupin (Krzyżosiak et al., 1988; Ciesiołka et al., 1989b). It is likely also that other tRNAs that contain C60 will show this property. On the other hand, none of the studied natural tRNAs that contain another base at position 60 was strongly cleaved in the D-loop by Pb(II) ions.

It has been suggested earlier, on the basis of crystallographic data of yeast tRNA^{Phe}, that there is insufficient room in the tertiary structure to accommodate the purine base at position 60 (Quigley at al., 1975). If this would be so, the presence of purine at this point in tRNA^{Phe} mutants would alter the local structure of tRNA and very likely prevent metal ion binding to that region. Having that possibility in mind, we looked closely at cleavages found in *in vitro* transcripts containing either a pyrimidine or a purine base at position 60. The inspection of Pb(II) and Eu(III) cleavage patterns in C60 variants showed that the U59U60 mutant behaved very much like both mutants with purine 60. The

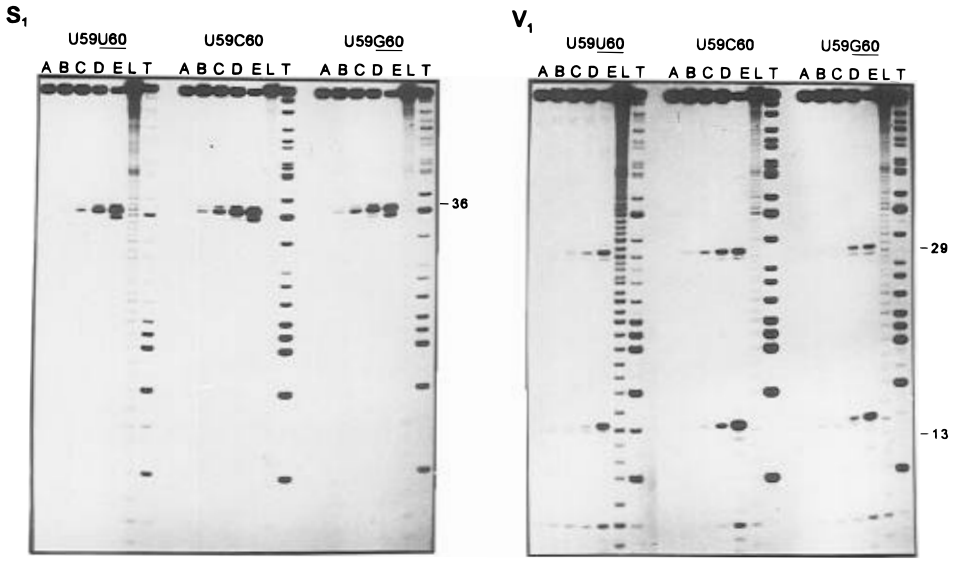

FIGURE 4: Time dependence of U59U60 mutant, wild type transcript, and U59G60 mutant hydrolysis by nucleases S_1 and V_1. Following time intervals B−E of 2, 5, 15, and 30 min for nuclease digestions were used. Lanes: A, reaction control; F, formamide ladder; and T, limited hydrolysis by RNase T_1.

	Pb^{2+}	Eu^{3+}	Mg^{2+}	Mn^{2+}
	16 21 G U U G G G A	16 21 G U U G G G A	16 21 G U U G G G A	16 21 G U U G G G A
U59C60 (wt)				
G59C60				
A59C60				
C59C60				
U59G60				
U59A60				
U59U60				

FIGURE 5: Distribution of metal ion-induced cleavages in the D-loop of yeast $tRNA^{Phe}$ wild type transcript and its U59C60 mutants. The mutated base is underlined, and numbering refers to phosphate residues. The thickness of the bars is correlated with the efficiency of cleavages.

efficiency of Pb(II) cuts was strongly reduced in all mutants. Also, the Eu(III)-induced cuts in the D-loop were suppressed, and in addition, their specificity was decreased. The results obtained with Eu(III) suggest that some tight binding of Eu(III) takes place in all mutants and that their binding sites have a very similar location as judged by the distribution of induced cleavages. This binding site, characteristic of mutants, is somewhat different from that present in the wild type transcript. On the other hand, the results obtained with lead(II) ions can be best explained by strong inhibition of Pb(II) binding to all mutants. This would be due either to the lack of sufficient room to bind the Pb(II) ion in that region or to the absence of right functional groups properly positioned to chelate the Pb(II) ion effectively.

In case of Mg(II)- and Mn(II)-induced cleavages, the $tRNA^{Phe}$ variants containing purine 60 and those with pyrimidine 60 form two pairs showing distinct cleavage patterns (Figure 5). The Mg(II)-induced cuts, although suppressed, clearly exist in U59A60 and U59G60 mutants, indicating that there must be some tight metal ion binding in the T-loop region. As concerns the Mn(II) ions, there is no question about the existence of high-affinity binding of Mn(II) ions to U59A60 and U59G60 mutants in which the cleavages are as strong as in pyrimidine 60-containing variants. The observed shift of the major D-loop cut in U59A60 and U59G60 mutants indicates that the binding site for these small ions is shifted in the way that the 2′-OH group of ribose 16 is most easily activated by the metal ion-bound hydroxyl. Thus, in U59A60 and U59G60 variants, the tight Eu(III), Mg(II), and Mn(II) ion binding in the T-loop is preserved but the D-loop cleavages occur with a slightly changed specificity. Although binding of the largest Pb(II) ion to the T-loop region cannot be definitely ruled out by the results presented in this paper, this possibility seems rather unlikely.

The analysis of known tRNA sequences shows that position 59 can be occupied by any of the four bases with the preference for adenine and uracil residues that were found in 48 and 31% of all tRNA sequences, respectively. As shown in this paper, the effect of mutation of uracil 59 on the efficiency of metal ion-induced cleavages is less profound than in the case of base changes at position 60. When U59 is replaced by purines, more specific hydrolysis takes place in the D-loop. This applies to all metal ions studied but

especially to Mg(II) and Mn(II) ions (Figure 5). In the case of these ions also, the efficiency of cuts increases in A59C60 and G59C60 mutants. On the other hand, the U59 to C59 mutation has only a minor effect on the specificity of D-loop hydrolysis which highly resembles that observed in the wild type transcript. These data give further support to our earlier proposal (Ciesiołka et al., 1989b) that, no matter what base is present at position 59, the efficient D-loop cleavage takes place when position 60 is occupied by cytosine.

In the crystal structure of yeast tRNA^{Phe}, the U59 and C60 residues are not involved in base stacking with other T-loop components but are rather bulged out and nucleate the perpendicular stacking system (Rich & RajBhandary, 1976). Both U59 and C60 are buried in the interior of the tRNA tertiary structure and remain inaccessible to chemical modification (Peattie & Gilbert, 1980; Holbrook & Kim, 1983). Also, chemical modification of the phosphate backbone with ENU showed significant protection of that region (Vlassov et al., 1981). Only the removal of wybuotine from the anticodon loop resulted in accessibility of C60 to chloroacetaldehyde modification (Krzyżosiak & Ciesiołka, 1983) and caused a significant increase in the efficiency of Pb(II)-induced cleavage at P18 (Krzyżosiak et al., 1988). The main reason why U59 and C60 in native yeast tRNA^{Phe} were resistant to chemical modification was probably the size of modifying reagents that were unable to enter the interior of the molecule. The U59 and C60 bases serve, however, as good ligands for metal ions which are smaller species that can penetrate that region, bind tightly, and promote specific cleavages (Figure 6).

Careful analysis of metal ion-induced hydrolysis shows also some structural changes in the T-stem and variable region. For example, in the case of the G59C60 mutant treated with Pb(II) ions, the phoshodiester bonds P63−P65 in the T-stem became reactive. The P64 was shown earlier to be one of the fragile sites of yeast tRNA^{Phe} structure, and the nearby phosphate P62 was another site of self-degradation of yeast and lupin tRNA^{Phe} and calf liver tRNA^{Trp} (Dock-Bregeon & Moras, 1987). The antibiotic bleomycin was also shown to cleave yeast tRNA^{Phe} at P64 and P62 (Keck & Hecht, 1996). In the case of the G59C60 mutant, either this site becomes altered in a way that favors the cleavage to be promoted by the Pb(II) ions acting from the solution, what seems less likely, or this cleavage is performed from a newly created Pb(II) ion binding site. A similar interpretation can be given to the Mg(II)- and Mn(II)-induced cuts at P61 in the wild type transcript and the C59C60 mutant, as well as to the Pb(II)-induced cleavages at P49 which are most profound in all C60 mutants. The latter cut was observed earlier in several other tRNA^{Phe} mutants, and the participation of the Pb(II) ion located at the Pb(1) site in this cleavage was ruled out (Behlen et al., 1990). It is also highly unlikely that the Pb(1) ion that induces the cleavages in the D-loop or Pb(2) could be responsible for the cut at P64 (Figure 6). In the yeast tRNA^{Phe} crystal structure, the distances from the Pb(1) and Pb(2) site to the 2′-OH group of ribose 63 that would have to be activated to induce the cleavage are 24.0 and 36.3 Å, respectively. The distance most favorable for the cleavage that occurs according to this mechanism is 6.0 Å (Brown et al., 1983). In spite of the changes discussed above, the structure alterations observed in U59C60 mutants of tRNA^{Phe}, although significant, are rather local. In favor of that conclusion are the results of enzymatic digestion. They

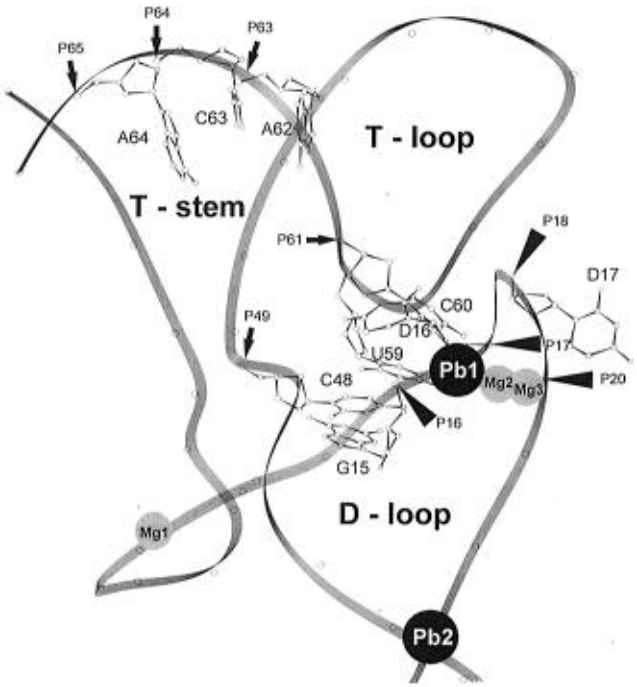

FIGURE 6: Metal ion binding and cleavage domain of yeast tRNA^{Phe}. Computer simulation of the tRNA structure was done by the INSIGHT II program (Biosym Technologies) using coordinates of the tRNA crystal structure deposited in the Brookhaven Protein Data Bank. Locations of the strongly bound lead and magnesium ions within the tRNA structure are according to Brown et al. (1985) and Jack et al. (1977). The major cuts induced by metal ions in the D-loop (triangles) and the minor cuts (arrows) are marked on the phosphodiester ribbon. Mutated nucleotides U59C60 and some nucleotides for which 2′-OH group activation is required to induce the observed cleavage of phosphate chain are shown. For simplicity, the C5′−O5′−P links and the G19 nucleotide are omitted.

show that double-strand-specific V_1 ribonuclease and single-strand-specific S_1 nuclease cannot distinguish between the wild type transcript and mutants. Thus, metal ions are better probes than the nucleases to reveal faint structural differences between tRNA variants.

In summary, we have described in this paper the cleavages induced in seven tRNA^{Phe} variants by four different metal ions. In other words, we have studied the cleavages in 28 tRNA mutant−metal ion pairs. This is one of the most comprehensive studies of a single metal ion binding "pocket" in tRNA. The wide spectrum of cleavage patterns observed can be considered as a source of new information regarding specific ligand requirements for different metal ions, and these data may contribute to a better understanding of the role played by metal ions in RNA catalysis.

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