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In Vitro Selection of RNAs That Undergo Autolytic Cleavage with Pb^{2+} †

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ABSTRACT: An in vitro selection method has been developed to obtain RNA molecules that specifically undergo autolytic cleavage reactions by Pb^{2+} ion. The method utilizes a circular RNA intermediate which is regenerated following the cleavage reaction to allow amplification and multiple cycles of selection. Pb^{2+} is known to catalyze a specific cleavage reaction between U17 and G18 of yeast $tRNA^{Phe}$. Starting from pools of RNA molecules which have a random distribution of sequences at nine or ten selected positions in the sequence of yeast $tRNA^{Phe}$, we have isolated many RNA molecules that undergo rapid and specific self-cleavage with Pb^{2+} at a variety of different sites. Terminal truncation experiments suggest that most of these self-cleaving RNA molecules do not fold like tRNA. However, two of the variants are cleaved rapidly with Pb^{2+} at U17 even though they lack the highly conserved nucleotides G18 and G19. Both specific mutations and terminal truncation experiments suggest that the D and T loops of these two variants interact in a manner similar to that of $tRNA^{Phe}$ despite the absence of the G18U55 and G19C56 tertiary interactions. A model for an alternate tertiary interaction involving a U17U55 pair is presented. This model may be relevant to the structure of about 100 mitochondrial tRNAs that also lack G18 and G19. The selection method presented here can be directly applied to isolate catalytic RNAs that undergo cleavage in the presence of other metal ions, modified nucleotides, or sequence-specific nucleases.

In the past two years, in vitro selection has became a powerful tool to isolate RNA molecules with a desired property from RNA pools with randomized sequences. In vitro selection has been applied to obtain specific ligands for proteins (Tuerk & Gold, 1990), organic dyes (Ellington & Szostak, 1990), the third strand of a triple helix (Pei et al., 1991), and catalytic RNAs derived from group I introns (Robertson & Joyce, 1990; Green et al., 1990). For catalytic RNAs, the selection procedures involve either cleavage of the substrate followed by ligation of the product (Robertson & Joyce, 1990) or ligation to a defined 5′ end of the catalytic RNA (Green et al., 1990). Since efficient ligation is prerequisite for these procedures, RNA molecules that catalyze cleavage reactions and do not reverse effectively cannot be easily selected. In addition, these selections are designed to carry out the reaction at a specific ribose phosphate bond; if the reaction were to occur elsewhere, the resulting variants are likely to be missed by these experimental strategies.

Yeast $tRNA^{Phe}$ undergoes a specific self-cleavage reaction when incubated with lead ion (Werner et al., 1976; Brown et al., 1985; Krzyzosiak et al., 1988; Behlen et al., 1990). The rate of Pb^{2+} cleavage has been shown to be very sensitive to the proper folding of the tRNA molecule. Single mutations that disrupt tertiary base pairs or triples reduce the cleavage rate by as much as 20-fold, while compensatory mutations that allow alternate tertiary interactions will restore the cleavage rate (Behlen et al., 1990).

We are interested in using the Pb^{2+} cleavage reaction in an in vitro selection experiment for two purposes: to isolate (1) novel RNA molecules that fold like tRNAs and (2) other RNAs that also undergo self-cleavage with Pb^{2+}. By using a selection protocol that involves cleavage of circular RNAs followed by enzymatic ligation at the cleavage sites, we were able to isolate RNA molecules that cleave specifically and rapidly at a variety of unique sites. Among these molecules were two variants which lack the highly conserved G18 and G19 in yeast $tRNA^{Phe}$, similar to some mitochondrial tRNAs.

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Materials and Methods

Preparation of Random RNA Libraries. The double-stranded DNA templates were made by annealing and extending two synthetic DNA oligonucleotides with 0.2 pmol of DNA/unit of MuLV reverse transcriptase at 37 °C for 1 h. For example, the sequences for the pool shown in Figure 2A are 5′TGGTGCGAATTCTGTGGATCGAACACAGGANNTCCAGATCTTCAGTCTGG and 5′TAATACGACTCACTATAACGGATTTNNCNNAGTTGGGANNGNGCCAGACTGAAGA, where the complementary sequences are underlined and N represents equal amount of the four nucleotides. Transcription by T7 RNA polymerase was carried out as previously described (Milligan & Uhlenbeck, 1989), except that a 5-fold excess of 5′-AMP over ATP was included in the transcription reaction to generate RNA with a 5′-monophosphate. In order to form an intramolecular circle, 10 μM linear RNA was incubated in 50 mM Tris, pH 7.6, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 0.2 mM ATP, 0.1 mg/mL BSA, 15% dimethyl sulfoxide, and 1 unit/μL T4 RNA ligase for 2 h at 37 °C. The circular RNA was purified from unreacted linear RNA on a 15% denaturing polyacrylamide gel (Pan et al., 1991).

The Selection Procedure. To initiate the cycle of selection, the gel-purified circular RNA was renatured by heating at 85 °C for 2 min in 15 mM MOPS, pH 7.0. After addition of $MgCl_2$ (15 mM for the first three selection rounds and 10 mM for the next three rounds), $Pb(OAc)_2$ was added to a final concentration of 0.4 mM and the reaction mixture incubated for 9 min at 22 °C. The reaction was terminated by addition of an equal volume of 9 M urea/50 mM EDTA, and the mixture was applied to a 15% denaturing gel to separate the lead-cleaved linear RNA product from the unreacted circular form. The linear RNA was eluted from the gel, ethanol precipitated, and then redissolved in 30 mM Tris, pH 8.0, 15 mM $MgCl_2$, and 1.5 units/μL T4 polynucleotide kinase, and the mixture was incubated for 45 min at 37 °C to remove the 2′,3′-cyclic phosphate (Weber, 1985). Upon addition of 30 mM Tris, pH 7.5, 8 mM dithiothreitol, and 40 μM ATP, the reaction mixture was further incubated at 37 °C for 15 min to introduce a 5′-phosphate. After the buffer was adjusted to the RNA ligase conditions described above, RNA ligase was added, and the mixture was incubated for 2 h at 37 °C. The reaction mixture was then extracted with an equal volume of phenol and precipitated with ethanol. The RNA was redissolved in 15 μL of 3.3 mM Tris, pH 7.5, and 0.33 mM EDTA. A primer of the sequence 5′TGGTGCGAATTCTGTGGA was added to 1 μM, and annealing was carried out by heating at 93 °C for 2.5 min followed by incubation on ice for 3 min. Reverse transcription was performed in 20 μL of 20 mM Tris, pH 8.3, 40 mM KCl, 0.2 mM dNTPs, and 4–6 units of AMV reverse transcriptase for 30 min at 37 °C. The RNA was degraded upon addition of 3 μL of 1 M NaOH and heating at 95 °C for 5 min. The resulting solution was neutralized with HCl and the cDNA precipitated with ethanol. PCR was performed in 40 μL including 4 μL (20%) of the cDNA under the following conditions: 95 °C, 30 s, 55 °C, 10 s, 72 °C, 2 min, 18 cycles, using the primer for reverse transcriptase and a second primer (5′TAATACGACTCACTATAACGGATT) containing the promoter sequence of T7 RNA polymerase. The reaction mixture from PCR was used directly in a 200-μL transcription reaction without purification.

Characterization of the Selected Pools of RNA. After six cycles of selection, the resulting cDNA was PCR amplified and purified on 12% nondenaturing polyacrylamide gels, taking care to avoid denaturation of the amplified double-stranded DNA. The DNA was then cloned into the *Sma*I site of pUC18 to allow selection on X-gal plates. Sixty-four white colonies were picked, and the plasmid DNA was sequenced using Sequenase 2.0 and protocols provided by the manufacturer (U.S. Biochemicals).

In order to test the individual variants for Pb^{2+} cleavage, plasmid DNAs were amplified by PCR using the same primers as described above, and RNAs were transcribed with T7 RNA polymerase in the presence of [α-^{32}P]CTP. The gel-purified linear RNAs were renatured by heating at 85 °C for 2 min in 15 mM MOPS, pH 7.0, and incubated in 10 mM $MgCl_2$ and 0.4 mM $Pb(OAc)_2$ for 6 min at 22 °C. The location of cleavage sites can be mapped using 5′ ^{32}P labeled molecules by comparing the Pb^{2+} cleavage products to the partial alkaline hydrolysis and ribonuclease T1 digestion products.

To determine the minimal lengths of RNAs that were still able to cleave with Pb^{2+} specifically, RNAs were 5′ ^{32}P labeled using [γ-^{32}P]ATP and T4 kinase or 3′ end labeled using [5′-^{32}P]pCp and T4 RNA ligase. Partial alkaline hydrolysis of end-labeled RNAs was performed at 1 μM RNA in 1 mM glycine and 0.4 mM $MgSO_4$, pH 9.5, by boiling for 2 min. The hydrolyzed RNA mixture was renatured at neutral pH, and Pb^{2+} cleavage was carried out in 30 mM MOPS, pH 7.0, 10 mM $MgCl_2$, and 0.4 mM $Pb(OAc)_2$ for 3 and 9 min at 25 °C. The resulting mixture was then analyzed on 8% denaturing polyacrylamide gels.

Mutations in RNAs were introduced by PCR amplification of plasmid DNA using primers complementary to the 3′ 30 nucleotides except for the sites of mutations followed by in vitro transcription with T7 RNA polymerase. The presence of mutations was confirmed by ribonucleases T1 and CL3 analysis of the in vitro transcribed RNAs from PCR-amplified templates.

Results

Selection of RNAs That Self-Cleave with Pb^{2+}. The selection procedure (Figure 1) involves preparing a randomized pool of RNAs by in vitro transcription of synthetic DNA templates by T7 RNA polymerase. The RNA is then circularized with T4 RNA ligase and gel purified. Following renaturation in the presence of Mg^{2+}, the circular RNA is cleaved with Pb^{2+}, and full-length linear molecules are separated from unreacted circular molecules on a denaturing gel. The linear RNAs are then recircularized by the combined action of T4 polynucleotide kinase and T4 RNA ligase and converted back into DNA templates by reverse transcriptase and PCR. The use of a circular RNA intermediate in the selection process makes it possible to select for Pb^{2+} cleavage sites between segments of random nucleotides.

Two different starting pools of RNAs were prepared which contained the yeast $tRNA^{Phe}$ sequence with random nucleotides at nine or ten positions that are involved in tertiary interactions in $tRNA^{Phe}$ (Figure 2). Although some of the variants are expected to form successful tertiary interactions and fold like $tRNA^{Phe}$ so that effective self-cleavage with Pb^{2+} at phosphate 18 will result, it is likely that the majority of the variants will not fold like tRNA, permitting a search for other Pb^{2+}-dependent self-cleaving RNAs. Gel purifications were carried out at three different stages of each selection cycle: purification of RNA from the transcription reaction, separation of circular RNA from unligated linear molecules, and separation of the Pb^{2+}-cleaved product from unreacted circular RNA. These separations ensured maximum efficiency of selection but also extended the time required for selection to

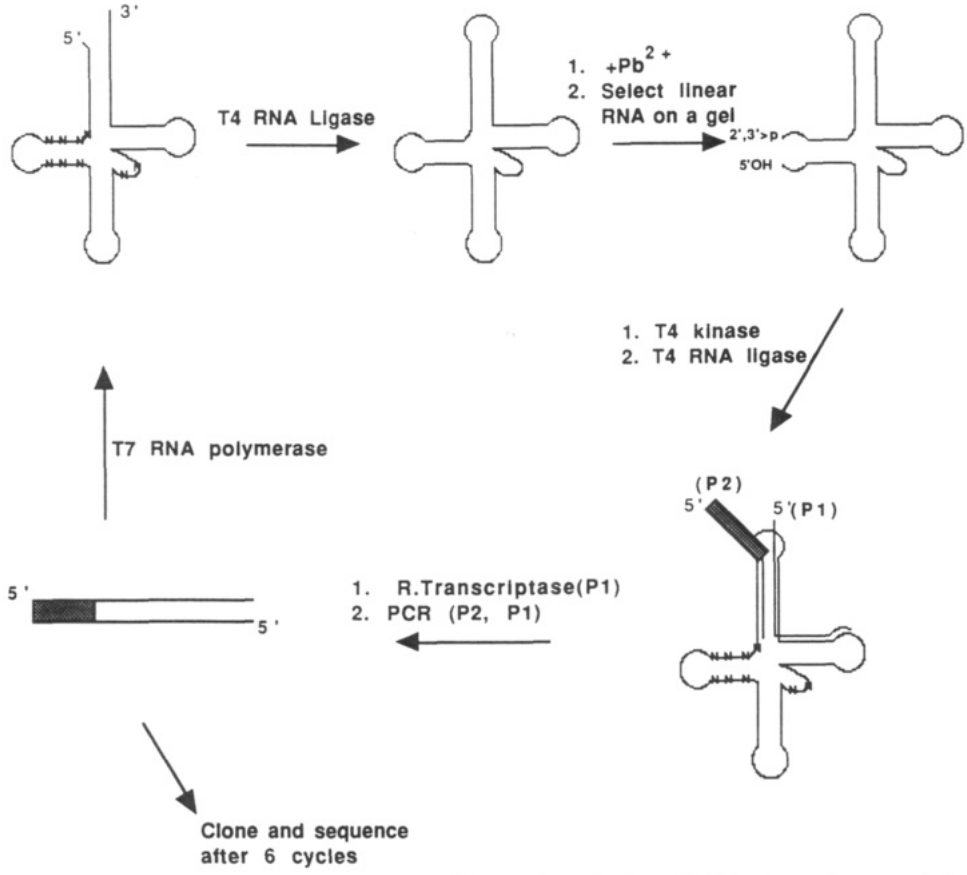

FIGURE 1: Selection of lead cleavage motifs: experimental design of the in vitro selection of RNAs that undergo autolytic cleavage with Pb^{2+}. The starting RNA pools are presented as cloverleaf secondary structure forms with positions randomized shown as N. The yeast $tRNA^{Phe}$ cleavage site is shown in the diagram, but cleavage at any other ribose phosphate position will also be religated to the circular form. P1 and P2 represent the DNA primers used to reverse transcribe and PCR amplify the selected RNA molecules. The filled box corresponds to the promoter sequence from −17 to −1 of T7 RNA polymerase.

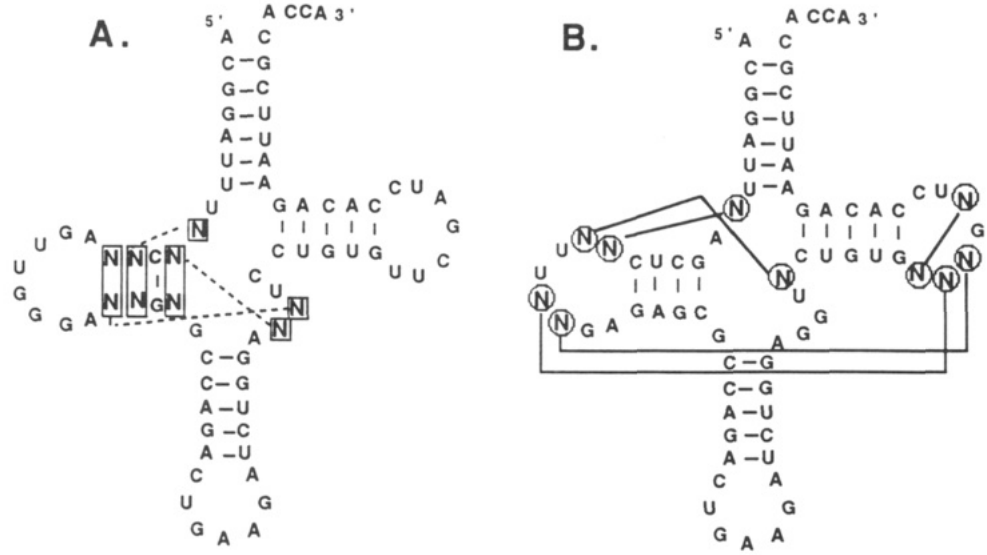

FIGURE 2: Starting pools of nine (A) or ten (B) randomized nucleotides shown as N in the background sequence of yeast $tRNA^{Phe}$. The boxes and dashed lines (A) and the circles and solid lines (B) correspond to the three base triplets and five highly conserved tertiary interactions in cytoplasmic tRNAs, respectively.

about 4 days per cycle. It should be pointed out that Pb^{2+} cleavage was not the only criterion for selection. Other steps in the selection cycle can have differing efficiencies with different RNA sequences. For example, since the expected Pb^{2+} cleavage products contained 5′-hydroxyl and 2′,3′-cyclic phosphate termini, we used T4 polynucleotide kinase to remove the cyclic phosphate at the 3′ end and to phosphorylate the 5′-hydroxyl group to generate appropriate functional ends for T4 RNA ligase. Since the efficiencies of both enzymes are dependent on the secondary structure of RNA (Richardson, 1981; Bruce & Uhlenbeck, 1978; Uhlenbeck & Gumport, 1982) and the RNA cannot be amplified without recircularization, our procedure also selects RNAs based on reactivity of the two enzymes at the cleavage site.

The Pb^{2+} cleavage conditions that give efficient cleavage of the yeast $tRNA^{Phe}$ transcript (Behlen et al., 1990) were

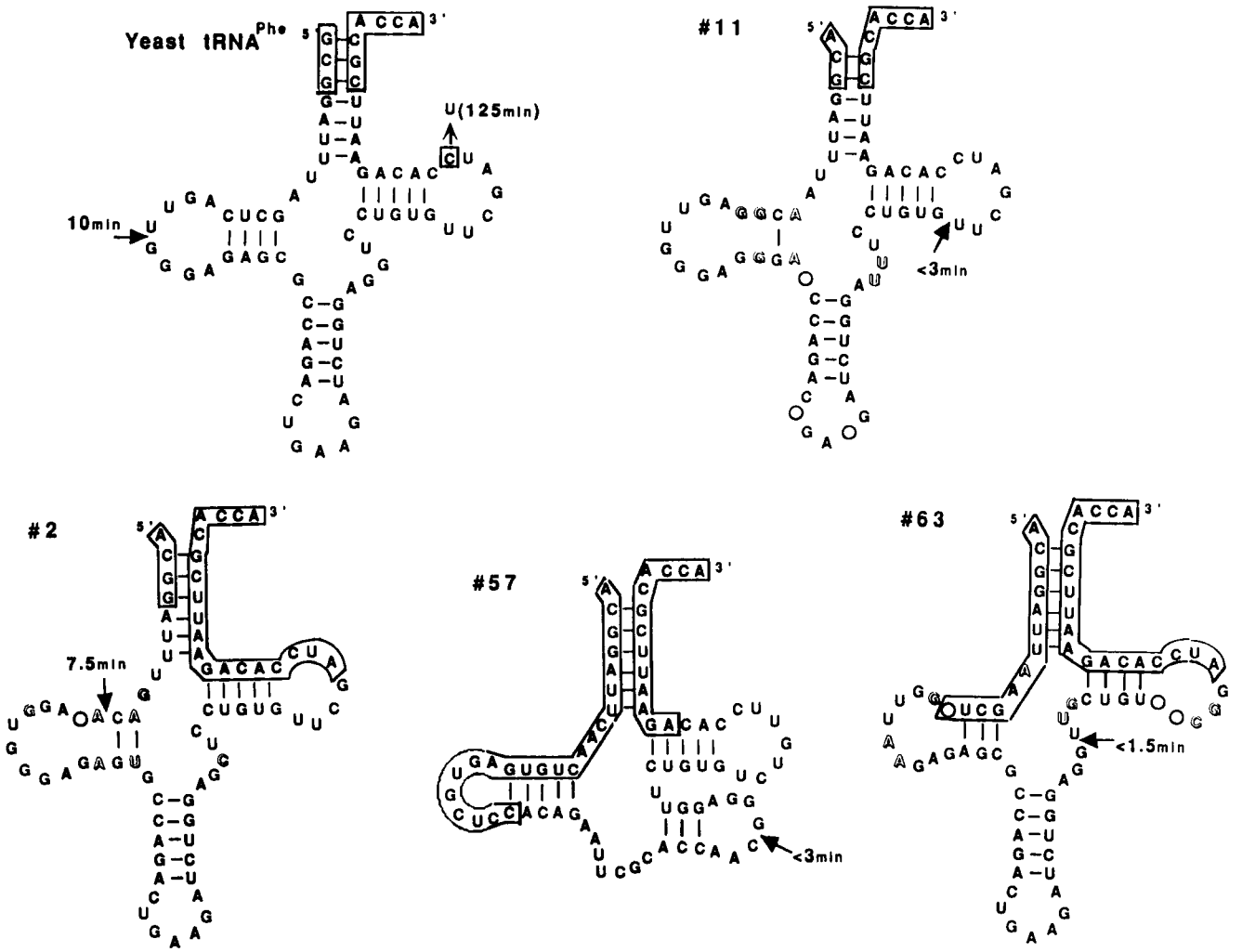

FIGURE 3: Cloverleaf presentation of some selected variants. The Pb^{2+} cleavage sites are shown by arrows. Numbers beside the arrows correspond to the half-life of the cleavage reaction at these sites (Table II). Nucleotides of these variants that are different from yeast tRNA^{Phe} are outlined, and deletions relative to yeast tRNA^{Phe} are shown as open circles. The boxes represent nucleotides that can be deleted without affecting the Pb^{2+} cleavage as determined by terminal truncation experiments. For yeast tRNA^{Phe}, the effect of C60U mutation is also shown (Behlen et al., 1990).

chosen to carry out the selection. The two starting pools were selected under otherwise identical conditions except that 1.5 mM spermine was included separately to generate a total of four reaction sets (Table I). The major difficulty of the selection protocol appeared to be random hydrolysis of the circular RNAs to produce linear RNA molecules which did not arise from cleavage by Pb^{2+}. Presumably due to this background problem, actual enrichment of the Pb^{2+}-cleaving RNAs occurred relatively slowly in the first three rounds. However, as shown in Table I, the extent of Pb^{2+} cleavage of the population of circular RNAs increased progressively in successive cycles. After six cycles of selection, all four populations of RNAs were cleaved by Pb^{2+} with the extent equal to or greater than that of pure circular yeast tRNA^{Phe}.

The pools after six cycles were amplified by PCR and cloned into pUC18 in *Escherichia coli*. Sixteen individual clones from each set were sequenced, giving a total of 62 clones containing insert sequences. Each plasmid DNA was amplified by PCR and transcribed by T7 RNA polymerase to produce linear RNA molecules to test for Pb^{2+} cleavage. Of the 62 clones tested in the presence or absence of spermine, 24 (39%) were cleaved by Pb^{2+} at a single site with rates similar to or faster than yeast tRNA^{Phe} (Table II). The remaining clones had slower cleavage rates (21/62) or multiple cleavage sites (12/62) or were RNAs that did not cleave with Pb^{2+} (5/62).

Table I: Extent of Pb^{2+} Cleavage of the Circular RNA[a] (%)

	set			
cycle	1	2	3	4
4	7.8	7.3	3.7	3.3
5	9.3	11.9	19.7	7.2
6	22.8	16.6	30.5	19.5

[a] Pb^{2+} cleavage is carried out in 15 mM MOPS, pH 7.0, 10 mM $MgCl_2$, and 0.4 mM $Pb(OAc)_2$ for 9 min at 23 °C. The extent of cleavage of the circular tRNA^{Phe} (G1A) is 20 ± 2% under identical conditions in the absence of spermine. The starting RNA pools for set 1/3 (Figure 2A) or 2/4 (Figure 2B) contain nine or ten randomized nucleotides. Conditions for all four sets are identical except that 1.5 mM spermine is included for set 3/4.

For the most clones tested, addition of 1.5 mM spermine appeared to have only marginal effects on either the rate or the site of cleavage (data not shown). It is obvious from Table II that, in addition to the expected sequence variations of the initially randomized nucleotides, most variants contained mutations, deletions, or insertions in the regions expected to be constant (see Discussion).

Many Distinct Pb^{2+} Cleavage Sites. Only 3 of the 24 isolates shown in Table II have Pb^{2+} cleavage sites located at or near the normal D loop site of yeast tRNA^{Phe}. The remaining 21 isolates are cleaved by Pb^{2+} at a variety of sites located throughout the RNA molecule. Although the sequences of

Table II: Sequences of Some Pb^{2+} Cleavage Domains from the Selection Experiment

Variant[a]	Sequence[b]	Apparent $t_{1/2}$ (min)[c]
	D stem / D loop / D stem / Anticodon loop / V loop / T stem / T loop	
	8 10 14 22 26 27 44 49 54	
$tRNA^{Phe}$	UA GCUC AGUUGGGA GAGC G CCAGACUGAAGAUCUGG AGGUC CUGUG UUCGA	10
Variants from the randomized sequence pool shown in Figure 2A.		
7*	UA *UCAA* AGU GA *UGGA* G CCAGACUGAAGAUCUGA AG*C*UC CUGUG U CGA	10
38*	U*G* *ACGG* AGUU A *AAGG* G CCAGACUGA*U*GAU UGG A*A*GUC CUGUG UUCGA	<2
55	UA *ACGG* A*U*UU*AACUAA* *UGGA* G CCAGACUGAAGAUCUGG A*A*GUC CUGUG UUCGA	
2*	U*G* *ACA* AG*G*UGGGA GAG*U* G CCAGACUGAAGAUCUGG AG*C*UC CUGUG UUCGA	7.5
37	*G* *ACAA* AGUUGGGA *UU*GC G CCAGACUGAA*A*AUCUGG AG*A*UC CUGUG UUCGA	
34*	U*G* *ACGG* AGUUGGGA *CCGA* G CCAGA UGAAGAUCUGG *GA*GUC CUGUG UUCGA	<1
42*	U*G* *ACG* AGUUGGGA *GGG* G CCAGACUGAAGAUCUGG A*A*GUC CUGUG UUCGA	
6	UA GC*GG* AGUUGGGA GAG G CCAGACUGAAGAUCUGG A*UU*UA CUGU UUC*AU*	
43	U*G* *ACGG* AG GGGA *CGGU* *U* CCAGACUGAAGAUCUGG A*C*AUC CUGUG UUCGA	
45	UA C*GG* AGUUGGGA *CU*GA G CCAGACUGAAGAUCUGG A*UU*UC CU UG U*AC*C*A*	
3	UA C*AG* AGU*A*GGGA *GGA* G CCAGACUGAAGAUCUGG AG*C*UC CUGUG UUCGA	
11*	UA *ACGG* AGUUGGGA G*GGA* CCAGAC GA GAUCUGG A*UU*UC CUGUG UUCGA	<3
59*	UG *GCGG* AGUUGGGA *AG*GC G CCAGACUGAAGAUCUGG A*U*GUC CUGUG UUCGA	
Variants from the randomized sequence pool shown in Figure 2B.		
53	*A*A GCUC *G*GU G*A*GA GAGC G CCAGACUGAAGAUCUGG A U*G* CUGAG *GU**U**GC*	
31	*G* GCUC *GU*UU*CU*GA GAGC *U* CCAG*G*CUGAAGAUCUGG AGGU*G* CUGUG UU*A*G*C*	
51	*AG* *AC*U*G* A*AGAUUAG* *AGGU* *A* C*U*AGACUGAAGAU UGG AGGU*A* CUGUG UU*UG**C*	
63*	*A*A GCU *G*GUU*AA*GA GAGC G CCAGACUGAAGAUCUGG AGGU*U* *G*CUGU *CG*GA	<1.5
66	*G* GCUC *GA*UUGGGA GAGC G CCAGACUGAAGAUCUGG AGGU*A* CUGU *CC*CGA	
28	*C*A GCUC *G*GUU*AC*GA GAGC G CCAGACUGAAGAUCUGG AGGU*U* CU UG U*CA*G	
Other RNAs that are cleaved specifically with lead[d]		
25,57*	CAA CUGUG AGUGCUC CACAG AAUUCGC ACC AACGGGA GGU U CUGUG UCUGU	<3
21	A	
30	Δ UGCGA A A	
60	G C Δ	

[a] The cleavage sites are indicated by an arrow. Nucleotides that are different from yeast $tRNA^{Phe}$ are drawn in italics and are underlined. Deletions are shown as blank space in the attempted alignment with the sequence of yeast $tRNA^{Phe}$. Asterisks indicate variants for which the cleavage sites have been determined precisely. If no asterisk is shown, the cleavage sites are estimated from [α-^{32}P]CTP-labeled RNA transcripts. [b] The sequences start from nucleotide 8 (tRNA nomenclature) and end at nucleotide 58. The 5′ nucleotides 1–7 (5′ACGGAUU) and 3′ nucleotides 59–76 (UCCACAGAAUUCGCACCA3′) are derived from PCR primers and hence are identical for all variants. [c] Cleavage conditions: 15 mM MOPS, pH 7.0, 10 mM $MgCl_2$, and 0.2 mM $Pb(OAc)_2$, 25 °C. The half-lives are calculated from the initial buildup rate of the cleaved products. [d] For variants 21, 30, and 60, only the changes in the nucleotide sequence to that of variants 25 and 57 are shown. The deletions are represented as Δ.

these isolates can differ from $tRNA^{Phe}$ by as few as seven nucleotides, many may not have secondary or tertiary structures similar to $tRNA^{Phe}$ (Figure 3). In addition to the new cleavage sites, the cleavage rates of several variants are at least 10-fold faster than that of $tRNA^{Phe}$ under identical conditions (Table II). The faster rates of cleavage of these variants may explain why sequences more closely resembling $tRNA^{Phe}$ were not found in the 62 variants. By any criteria $tRNA^{Phe}$ would appear to be only one of many RNAs that self-cleave at intermediate rates. In the previous studies no single, double, or triple mutants were found that increased the Pb^{2+} cleavage rates by more than 1.4-fold (Behlen et al., 1990).

Although the number of nucleotides in all variants is similar to that of yeast $tRNA^{Phe}$, the minimal size needed for effective

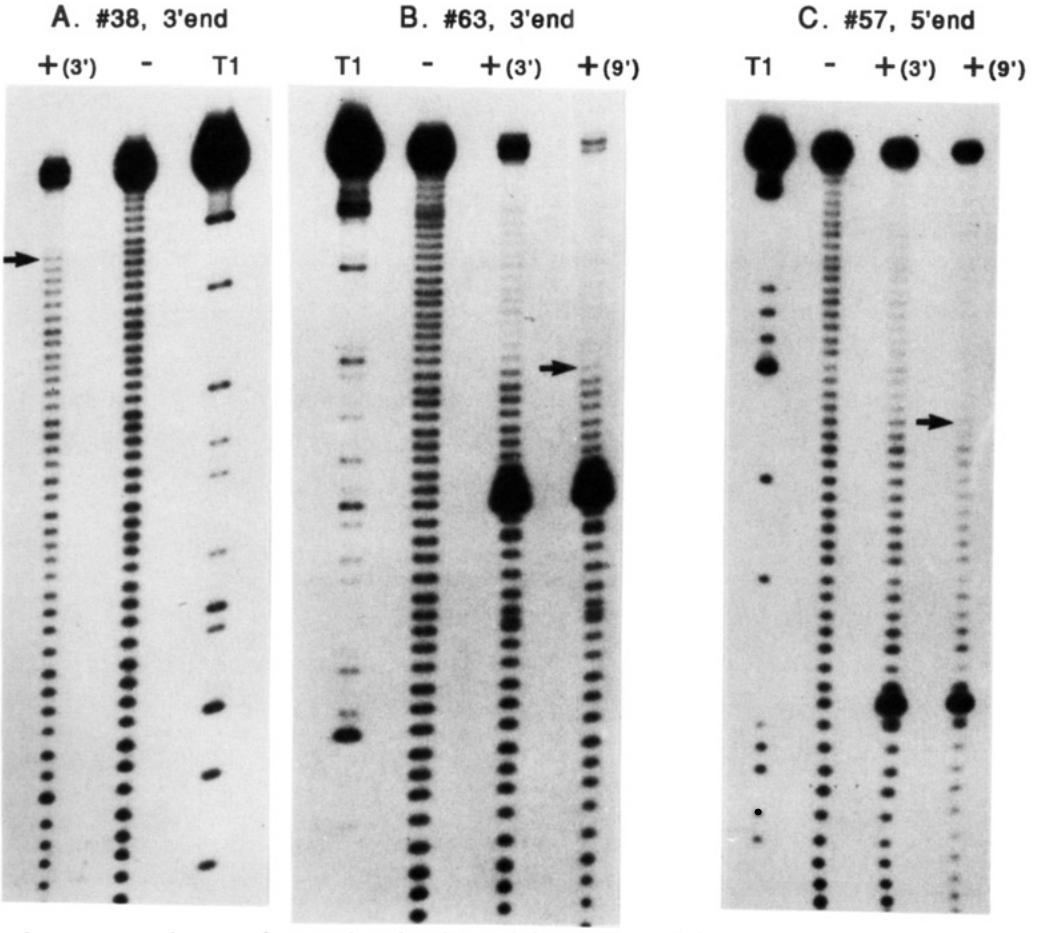

FIGURE 4: Minimal sequence requirement of some selected variants: (A) variant 38, minimal 3′-end determination; (B) variant 63, 3′ end; (C) variant 57, 5′ end. Symbols: −, partial alkaline hydrolysis of the ^{32}P end labeled molecules only; +, alkaline hydrolysis followed by cleavage with Pb^{2+} (the numbers in parentheses indicate the cleavage reaction times in minutes); T1, partial ribonuclease T1 digest of the same RNA. The end limits are indicated by arrows; they are deduced from comparison of band intensities to the control (−) and the partial T1 digestion.

Pb^{2+} cleavage differs substantially among the variants. By carrying out Pb^{2+} cleavage after partial alkaline hydrolysis of ^{32}P end labeled RNA molecules and analyzing the reaction mixture on high-resolution denaturing gels, the 3′ and 5′ boundaries of the Pb^{2+} cleavage motif can be established. As shown in Figure 4, all partial alkaline hydrolysis products which cleave with Pb^{2+} will be diminished with respect to an uncleaved control. The results of several of such end mapping experiments are summarized in Figure 3. For yeast $tRNA^{Phe}$ and variant 11, the acceptor stem is required for effective cleavage (Figure 3). However, the cleavage site in variant 11 is between G53U54 in the T loop, a distance of 22 Å from the U17G18 site in $tRNA^{Phe}$. For variants 2, 57, and 63, much less of the RNA is needed for Pb^{2+} cleavage (Figure 3), suggesting that they possess very different folding motifs.

Two tRNA-like Variants. Two variants, 7 and 38, are cleaved by Pb^{2+} in a D loop that contained only five nucleotides (Figure 5, Table II). The deletions in the D loop include G18 and G19 in yeast $tRNA^{Phe}$, two conserved nucleotides which form tertiary interactions with U55 and C56 in the T loop. In variant 7, the nucleotide at position U55 in the T loop is also missing. In addition to the deletions, the D stems of both variants contain at least one A-G or G-A opposition which are among the most stable non-Watson–Crick base pairs (SantaLucia et al., 1990, 1991). Despite these differences, several lines of evidence suggest that variants 7 and 38 maintain a folded tertiary structure similar to that of tRNA. First, like $tRNA^{Phe}$, most of the acceptor stems of variants 7 and 38 must be intact for rapid Pb^{2+} cleavage (Figures 4 and 5). Second, when the C60U mutant is made in variant 38, the lead cleavage rate was reduced 16-fold, while the cleavage site in the D loop was unaltered (Figure 5B). Since C60 in yeast $tRNA^{Phe}$ is involved in coordinating the Pb^{2+} (Rubin & Sundaralingam, 1983; Brown et al., 1985) and the same C60U mutant reduces the Pb^{2+} cleavage rate 12.5-fold (Behlen et al., 1990), the result suggests that the lead ion in variant 38 is coordinated at the same site as in yeast $tRNA^{Phe}$ and that a similar D loop–T loop interaction occurs. Finally, when U55 was restored in variant 7 to give it the same T loop sequence as in yeast $tRNA^{Phe}$, the Pb^{2+} cleavage rate in the D loop was reduced 30-fold (Figure 5A). This is again suggestive of the presence of essential loop–loop interactions for proper tertiary folding of variant 7.

The structure of yeast $tRNA^{Phe}$ contains two interloop tertiary interactions, G18U55 and G19C56 (Figure 5C). Previous studies have shown that both of these tertiary interactions are essential for effective Pb^{2+} cleavage and aminoacylation with phenylalanyl-tRNA synthetase (Behlen et al., 1990; Sampson et al., 1990). To maintain a tRNA-like structure without G18 and G19, variants 7 and 38 presumably replace G18U55 and G19C56 with other tertiary interactions. Since it is reasonable to suggest that both variants maintain the other tertiary interactions found in yeast $tRNA^{Phe}$ (U8A14, G15C48, and U54A58), only U16, U17, U55, and C56 remain available to establish tertiary contacts between the loops.

In an attempt to rationalize the rapid cleavage of variant 38 with Pb^{2+}, a molecular model was built. Since variant 38 appears to contain the same Pb^{2+} binding site as yeast

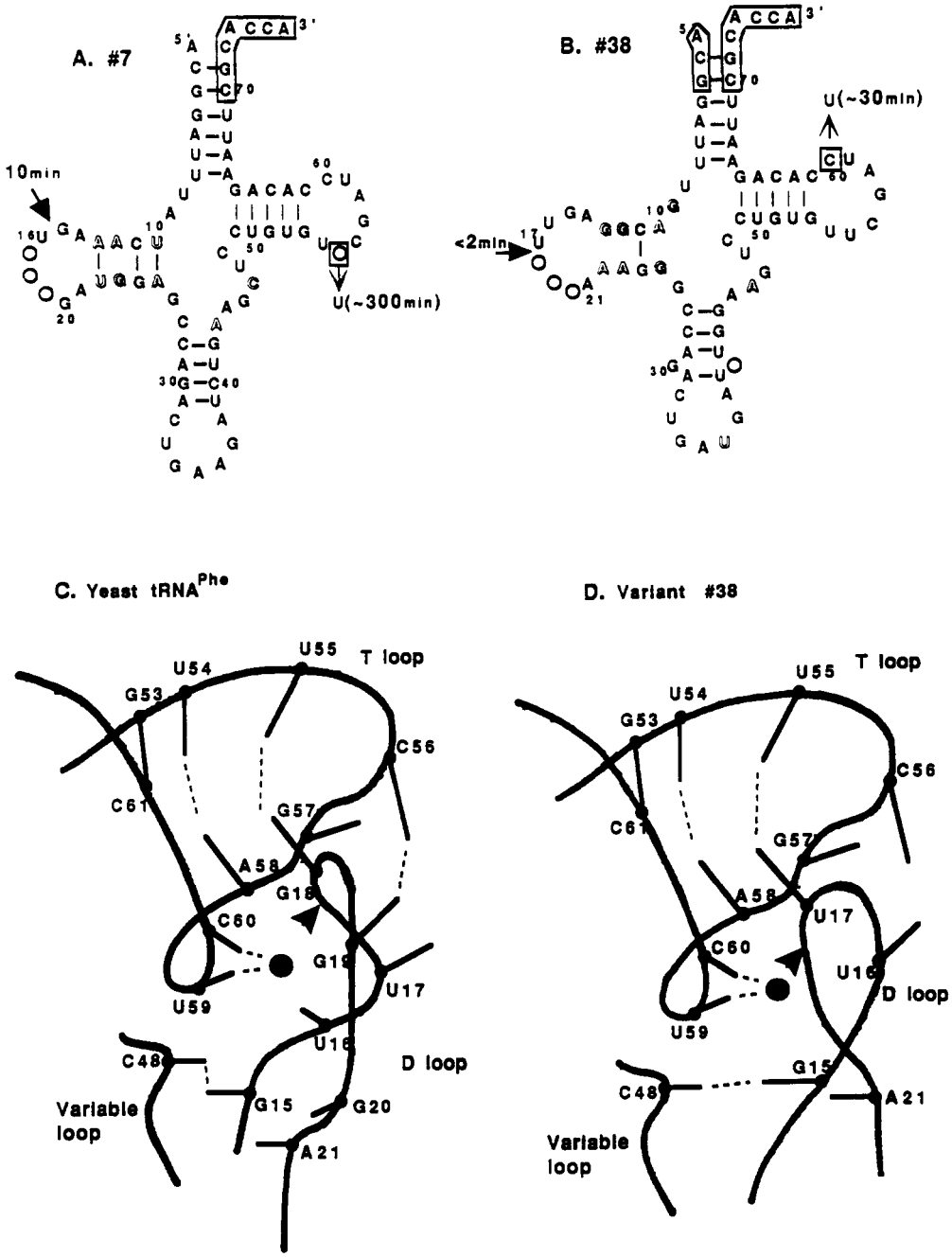

FIGURE 5: Cloverleaf presentation of variants 7 (A) and 38 (B). The RNAs are numbered using standard tRNA nomenclature. Symbols used are the same as described in the legend of Figure 3. (C) D loop–T loop interactions of yeast $tRNA^{Phe}$, adapted from the crystal structure where the phosphodiester backbone is represented by a ribbon and the bases are represented as solid lines. Dashed lines indicate the tertiary interactions of nucleotide bases. Pb(1) is shown as a solid sphere, and the site of cleavage is indicated by an arrowhead. (D) Proposed folding model for variant 38. The same symbols are used as in panel C.

$tRNA^{Phe}$, we hypothesized that the backbone conformation of the T loop was very similar to that of $tRNA^{Phe}$. After removing residues 18–20 and connecting the U17–A21 phosphodiester bond, we placed the 2′-hydroxyl of the ribose of U17 in reasonable proximity [6 Å; see Brown et al. (1985)] to the bound Pb^{2+} to permit cleavage. With these constraints, it was not possible to form either U16U55 or U17C56 tertiary interactions, but several favorable pairing arrangements between U17 and U55 could be proposed (Figure 5D). If one further restricted the base and ribose conformation of U55 to be that of $tRNA^{Phe}$, U17 can be positioned such that three hydrogen bonds could be formed, two between the uridine bases and one between either O^2 or O^4 of U17 and the 2′OH of U55. Formation of U-U base pairs is not unprecedented (Gutell & Woese, 1990) and has been found to be stabilizing within RNA helices (SantaLucia et al., 1991). Beside U17U55, additional tertiary contacts involving U16 or C56 may also exist.

Discussion

We have described a method for selection of RNAs capable of specific cleavage of the phosphodiester bonds from a large population of molecules. By use of a circular RNA intermediate before and after the cleavage reaction, the protocol does not restrict cleavage to any particular site in the molecule. This protocol could be easily adapted to isolate RNAs that undergo autolytic cleavage in the presence of (1) other metal ions such as Zn^{2+} or Mn^{2+} or (2) cofactors such as modified nucleotides. Sequence requirements for specific cleavage reactions by ribonucleases can also be deduced using the same

method. A similar protocol could be developed to study recognition sites for sequence-specific deoxyribonuclease cleavage reactions.

It is important to emphasize that factors other than specific cleavage may influence the outcome of the selection. The use of polynucleotide kinase and RNA ligase to re-form circles after cleavage means that their efficiency at a particular site is part of the selection processes. Since the rate of joining by T4 RNA ligase is substantially influenced by the sequence, secondary structure, and proximity of the two termini (Uhlenbeck & Gumport, 1982), this is potentially a very strong selective pressure. This may explain why most of the Pb^{2+} cleavage sites appeared in potentially single-stranded regions of the molecule. In addition, the selection method does not distinguish single or multiple cleavage sites in the same molecule. Since selection is carried out under partial digestion conditions, molecules having multiple cleavage sites will accumulate according to the sum of cleavage rates at all sites. A combination of these effects could explain why only 24 of 62 clones have cleavage rates similar to or faster than that of yeast $tRNA^{Phe}$ (Table II), although the extent of cleavage of the RNA pools already reaches or exceeds that of the $tRNA^{Phe}$ (Table I). Had we carried out the selection for more cycles, a higher percentage of fast-cleaving domains might have emerged.

While the starting libraries used in this work restricted the random nucleotides to a relatively limited number of well-defined positions in the molecule, it is evident from the sequences of the selected variants (Table II) that mutations have been introduced in the nonrandomized regions in the molecule during successive cycles, thus enlarging the total number of variants being selected. The mutations are probably due to infidelity of T7 RNA polymerase, AMV reverse transcriptase, and *Taq* DNA polymerase. Deletions can be introduced by double cleavage of the ribose phosphate backbone of the same molecule, either by Pb^{2+}, by reversal of T4 RNA ligase (Krug & Uhlenbeck, 1982), or by trace amounts of contaminating nucleases in the enzymes. Since the combined action of T4 kinase and RNA ligase can repair cleavage by any of the above mentioned reactions, the resulting RNA molecules can be carried over into the next cycle of selection.

Due to the low pK_a value of an inner sphere coordinated water, Pb^{2+} is an effective RNA cleavage reagent by deprotonation of the 2′OH groups (Farkas, 1968, 1975). However, under the conditions used in this work, the Pb^{2+} concentration is sufficiently low and competing Mg^{2+} concentration sufficiently high that rates of random cleavage of RNA by Pb^{2+} are quite slow. Specific cleavage by Pb^{2+} is achieved by tight binding of Pb^{2+} to particular sites on the RNA molecule. In the case of yeast $tRNA^{Phe}$, three high-occupancy Pb^{2+} binding sites have been located by X-ray crystallography (Rubin & Sundaralingam, 1983; Brown et al., 1985). Only one of the three, Pb(1), causes the specific cleavage both in solution (Werner et al., 1976) and in the crystal (Brown et al., 1985). From a careful analysis of the three Pb^{2+} sites, Brown et al. (1985) concluded that, in order to facilitate RNA backbone cleavage, (1) the Pb^{2+} must be placed in the correct position with respect to a 2′-hydroxyl and (2) the region of the molecule where cleavage occurs must be sufficiently flexible for the structure to adjust to a conformation suitable for cleavage. Of the three sites in $tRNA^{Phe}$, only Pb(1) meets both requirements. The distance between Pb(1) and the 2′OH of ribose 17 is 6.0 Å, and although Pb(1) resides in a fairly immobile pocket formed by U59 and C60, the sugar phosphate backbone of residues 16–18 shows a large temperature factor in the crystal and is likely to be flexible in solution.

The large number of new Pb^{2+} cleavage sites found among the RNA variants in this work can be understood in two ways. First, the sequence changes may alter the conformation and flexibility of the $tRNA^{Phe}$ backbone sufficiently to permit cleavage by Pb(1) at new sites or by Pb(2) or Pb(3). Indeed, some natural tRNAs show comparatively fast cleavage rates in the anticodon loop (Werner et al., 1976), presumably due to Pb(3). Cleavage at a "cryptic" site (ribose phosphate 48) in several mutants of yeast $tRNA^{Phe}$ may result from an altered RNA conformation or the creation of a new Pb^{2+} binding site (Behlen et al., 1990). Thus, cleavage of variant 11 at G53U54 may be the result of Pb(1) cleaving an altered structure, cleavage of variant 63 at G46U47 may be caused by Pb(2), and cleavage of variant 31 at A38U39 in the anticodon loop may be due to Pb(3).

A second potential explanation for the large number of new Pb^{2+} cleavage sites is that the mutant RNAs contain binding sites for Pb^{2+} that are either entirely new or sufficiently improved to result in rapid cleavage. Undoubtedly, some of the variants fold quite differently from $tRNA^{Phe}$ so that new structures are formed. The ability of RNA to form a variety of different specific Pb^{2+} binding and cleavage sites is supported by the large number of cleavage sites in 16S rRNA (Gornicki et al., 1989) and in the chicken β-tropomyosin pre-mRNA (d'Orval et al., 1991).

It is quite striking that, of the only three variants that cleaved in the "expected" D loop site, two of which also contained deletions that removed the highly conserved residues, G18 and G19. Since in both cases T loop mutation substantially reduced the Pb^{2+} cleavage rate (Figure 5A,B), it appears that the D and T loops still interact in these molecules despite the fact that the G18U55 and G19C56 tertiary interactions are destroyed. For one of the variants, we proposed that a new U17U55 tertiary interaction was used to stabilize the loop–loop interaction.

There are nearly 100 mitochondrial tRNAs that have D and T loop structures similar to those of variants 38 and 7 [from the sequence collection of Sprinzl et al. (1989)]. The D loops of these tRNAs all lack G18 and G19 and generally contain five residues while the T loops mostly contain seven or six residues. The major differences between variant 38 and these mitochondrial tRNAs are (1) the D stems of mitochondrial tRNAs consist of Watson–Crick base pairs instead of the A-G pairs in variants 38 and 7 and (2) the variable loops in mitochondrial tRNAs have four nucleotides instead of five residues present in variants 38 and 7. These mitochondrial tRNAs must have the characteristic L-shaped tertiary structure, since other tRNAs in the same organelle have ordinary (cytoplasmic) nucleotides involved in tertiary interactions. This conclusion is also true for variants 38 and 7 based on our Pb^{2+} cleavage data.

To what degree is the model we proposed for variant 38 (Figure 5D) relevant to these mitochondrial tRNAs? The phylogeny of mitochondrial tRNAs which have U8A14, A15U48, and A21, 5, 4, and 7 residues in the D, V, and T loops, respectively, does support the proposal that G18U55 and G19C56 may also be replaced by a U-U tertiary interaction. Of the 23 mitochondrial tRNAs of this class, 13 (57%) contain U17U55 and therefore resemble variant 38. The remaining combination at these positions, U17C55 (2), U17A55 (1), C17U55 (2), A17U55 (3), G17U55 (1), and C17C55 (1), also shows preference of retaining a U at these two positions. Alternate base pairings involving uridine have been characterized in the structures of UUCG tetraloops (Cheong et al.,

1990) and a RNA duplex with a U-C pair (Holbrook et al., 1991).

It is possible that the sequence differences between variant 38 and the mitochondrial tRNAs reflect alternate folding strategies to ensure close contact between the T loop and the smaller D loop. Since it is known that the "propeller-twisted" G26-A44 pair in $tRNA^{Phe}$ causes the distance between the base pairs above and below to increase slightly, the consecutive G-A pairs in variant 38 may also increase the distance between the anticodon stem and the smaller D loop to permit better interaction of the D loop with the T loop. In the mitochondrial tRNAs, the same structural juxtaposition may be achieved by the shorter variable loop. The shorter variable loop may induce a tilt in the D stem with respect to the T stem, allowing closer contact between the D and T loops, as observed in the crystal structure of yeast $tRNA^{Asp}$ (Moras et al., 1980; Westhof et al., 1985).

Further information will be required to establish the precise folding of this class of mitochondrial tRNAs. Nevertheless, it is interesting that a selection procedure designed to provide information about the folding of cytoplasmic tRNAs resulted in sequences relevant to the folding of a distinct class of mitochondrial tRNAs.

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