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Lead-catalyzed cleavage of ribonuclease P RNA as a probe for integrity of tertiary structure

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ABSTRACT

Pb^{2+}-catalyzed cleavage of RNA has been shown previously to be a useful probe for tertiary structure. In the present study, Pb^{2+} cleavage patterns were identified for ribonuclease P RNAs from three phylogenetically disparate organisms, *Escherichia coli*, *Chromatium vinosum*, *Bacillus subtilis*, and for *E.coli* RNase P RNAs that had been altered by deletions. Each of the native RNAs undergoes cleavage at several sites in the core structure that is common to all bacterial RNase P RNAs. All the cleavages occur in non-paired regions of the secondary structure models of the RNAs, in regions likely to be involved in tertiary interactions. Two cleavage sites occur at homologous positions in all the native RNAs, regardless of sequence variation, suggesting common tertiary structural features. The Pb^{2+} cleavage sites in four deletion mutants of *E.coli* RNase P RNA differed from the native pattern, indicating alterations in the tertiary structures of the mutant RNAs. This conclusion is consistent with previously characterized properties of the mutant RNAs. The Pb^{2+} cleavage assay is thus a useful probe to reveal alteration of tertiary structure in RNase P RNA.

INTRODUCTION

Ribonuclease P (RNase P) cleaves leader sequences from pre-tRNAs to generate mature 5′ ends. The bacterial RNase P holoenzyme consists of a 130 kDa RNA subunit and a 14 kDa protein subunit. However, at high ionic strength *in vitro* the RNA subunit is catalytically active in the absence of the protein (1). Key to understanding the function of this ribozyme is detailed knowledge of its structure. Secondary structure models for the bacterial-type RNase P RNAs have been proposed based on phylogenetic comparisons (2, 3, Fig. 1). Despite extensive sequence dissimilarities, the RNase P RNAs from different organisms contain a conserved core of homologous sequence and secondary structure (2). Within the conserved core are regions of yet-unknown structure (shown in Fig. 1 as unpaired), which are likely involved in tertiary interactions.

Mutational studies are proving to be useful in defining structure–function relationships in RNase P RNA (e.g. 3–6). However, interpretation of results with mutant RNAs can be difficult. For instance, a mutation that influences global structure could have large effects on catalytic properties, even if the mutation were distant from the site of catalysis and played no direct role in catalysis. It was useful, therefore, to establish an assay for the integrity of the tertiary structure of RNase P RNA.

Previous studies have shown Pb^{2+}-promoted cleavage of RNA to be a useful assay for probing tertiary structure in $tRNA^{Phe}$ and its mutants (7–9). Pb^{2+}-catalyzed cleavage of RNA requires tight binding of Pb^{2+} at a precise orientation, dictated by tertiary structure, and cleavage results in the formation of 2′,3′-cyclic phosphate and 5′-hydroxyl termini (10, 11). We applied the Pb^{2+}-cleavage assay to RNase P RNA in an effort to identify a probe for intact tertiary structure in mutant RNAs. Comparisons of Pb^{2+}-promoted cleavage patterns of wild-type and mutant RNase P RNAs show that Pb^{2+} cleavage is an effective method for identification of structural disruptions in mutant RNAs.

MATERIALS AND METHODS

Preparation of RNA

RNAs were prepared *in vitro* by T7 RNA polymerase (B.Pace, Indiana University) transcription of plasmids EcP2 (*Escherichia coli*), DW66 (*Bacillus subtilis*), AB12 (*Chromatium vinosum*), EcP2Δ1 (Δ1), EcP2Δ4 (Δ4), SD10 (Δ2Δ3); tRNAs were transcribed (12) from plasmids DW 152 (*B.subtilis* pre-$tRNA^{Asp}$) and 67CF0 (*E.coli* $tRNA^{Phe}$), which have all been described (4, 5, 9, 13). Transcripts were purified by electrophoresis through 6% polyacrylamide gels containing 7M urea in 1×TBE buffer (90 mM Tris–HCl pH 8.0, 90 mM boric acid, 2 mM EDTA), visualized by UV shadow, passively eluted in TE (10 mM Tris–HCl pH 8.0, 1 mM EDTA) containing 0.1%

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SDS, recovered by ethanol precipitation, and dissolved in 10 mM Tris pH 7.4. RNA concentrations were determined by absorbance at 260 nm.

Pb^{2+}-catalyzed cleavage reactions

A typical 10 μl cleavage reaction contained 5 pmol of RNase P RNA in standard reaction buffer consisting of 10 mM Tris pH 7.5, 10 mM $MgCl_2$, and 100 mM KCl. Pb^{2+} concentrations varied from 0.05 mM to 400 mM. In standard buffer, optimal cleavage was observed between 0.1 mM and 0.5 mM $Pb(OAc)_2$. RNAs were pre-incubated for 5 minutes at 30°C in reaction buffer before the addition of $Pb(OAc)_2$. After 30 minutes at 30°C, reactions were stopped by addition of EDTA to a final concentration of 20 mM or, at higher concentrations of Pb^{2+}, to a final concentration equivalent to that of the Pb^{2+}. Cleavage products were recovered by ethanol precipitation and dissolved in TE buffer.

Primer extension analysis

In a typical primer extension analysis (13), 1 pmol of RNase P RNA was hybridized to 0.1–0.2 pmol of 5′-^{32}P-labeled oligodeoxynucleotide primer (50 mM K·HEPES pH 7.0, 25 mM KCl, 5 mM K·borate pH 7.0, at 90°C for 2 min followed by slow cooling to <40°C). The following primers were used for *E.coli* and deletion mutants: TGCGCGGGCCATCGGCGG (142R), GGGTGGAGTTTACCGTGC (225R), ACTGAICG-ATAAGCCGGG (352R); for *B.subtilis*: CTTCGCTAGGWA-CGAACACT (80R), TCTAGTGAGACTTCGTC (189R), AAATTTGGGWWWCTCGCTCGA (235R), CTACAGAAA-GCATTCTGTCCTTCTCTCCGTTGA (282R), GTGGTCTAA-CGTTCTGT (382R); for *C.vinosum*: CGATGAAGGGAGT-CGGCC (355R), CTCTTACCSCACCNTTTCACCCT (174RN). This collection of primers allowed analysis of all nucleotides in each RNA except for the 3′-terminal ca. 20 nucleotides, which are largely involved in known helical structure. Primer extension was carried out by AMV reverse transcriptase (Seikagaku, MD) in 50 mM Tris–HCl pH 8.5, 50 mM KCl, 10 mM DTT, 10 mM $MgCl_2$, 150 μM each dNTP at 42°C for 30 min. For sequencing reactions, dideoxynucleotide was added to a concentration of 25 μM. Reactions were stopped by addition of 2.5 volumes ethanol and 0.1 volume 3 M NaOAc for precipitation. Products were resolved by electrophoresis through 6% polyacrylamide containing 7M urea in 1×TBE buffer.

RESULTS

Pb^{2+} cleavage of *E.coli* RNase P RNA occurs in the conserved core

Sites of Pb^{2+}-catalyzed cleavage were identified by primer extension analysis of RNase P RNAs exposed to $Pb(OAc)_2$ under the conditions described (see Materials and Methods). Figure 2 shows representative primer extension analyses. Pb^{2+} catalyzes cleavage of *E.coli* RNase P RNA (Fig. 2A) mainly at nine positions (summarized in Fig. 1), with minor cleavages occurring at four additional sites (before nucleotides G_{120}, A_{266}, A_{268} and U_{303}). Bands that occur independently of Pb^{2+} cleavage correspond to pauses by reverse transcriptase on the highly structured RNase P RNA template. At the higher concentrations of Pb^{2+} tested (Fig. 2A), some cleavage sites diminish, perhaps because of structural distortion induced by that ion. The nine major cuts all occur in the conserved core of the RNA, the collection of sequence and secondary structural elements present in all bacterial RNase P RNAs (14). No cleavage sites are observed in proposed helical regions: all thirteen occur in regions of yet-unknown, possibly tertiary, structure. This limitation of

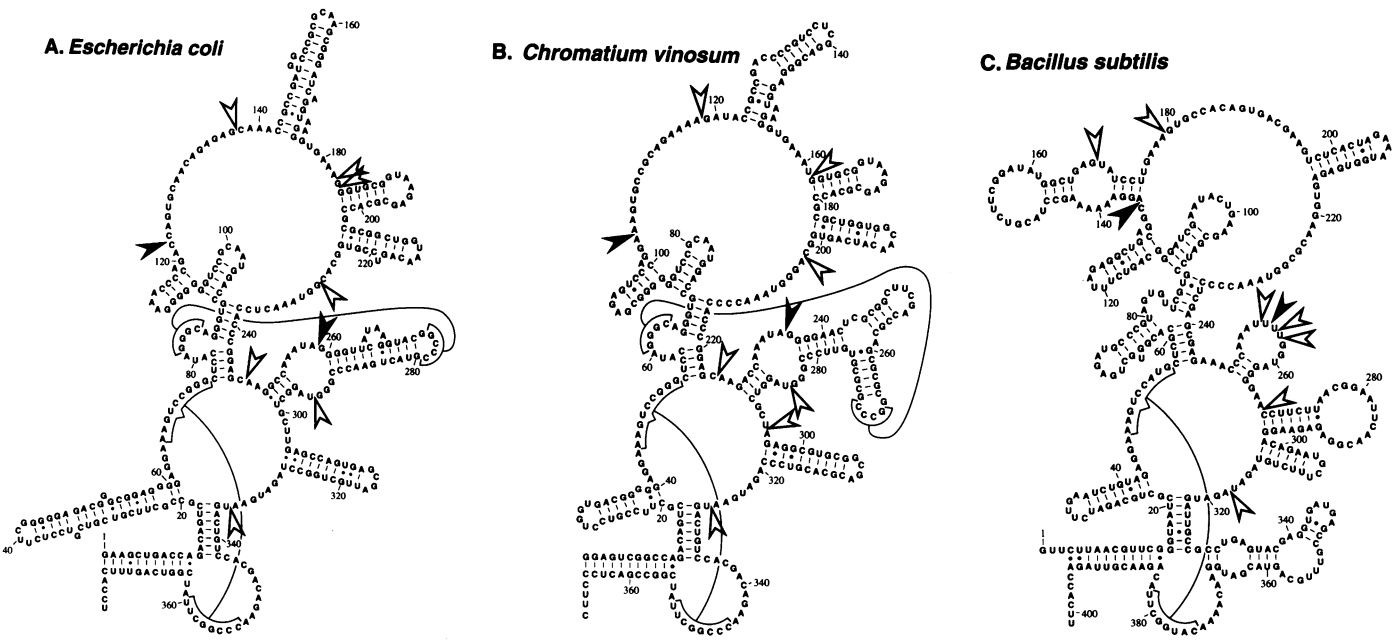

Figure 1. Pb^{2+}-catalyzed cleavage sites in RNase P RNAs. Secondary structure models of *E.coli*, *C.vinosum*, and *B.subtilis* RNase P RNAs are shown. The sites of Pb^{2+}-catalyzed cleavage inferred from primer extension analysis are indicated with arrows. Dark arrows indicate the major sites of cleavage shared by all three organisms at homologous nucleotides.

Pb^{2+} cleavage sites to regions of possible tertiary structure is also seen with the core structure of group I intron RNA (15) and with 16S rRNA (A.Hüttenhofer, unpublished results). Cleavage sites in the RNase P RNA are the same at elevated salt concentrations (Materials and Methods), under which the ribozyme is maximally active, although higher concentrations of Pb^{2+} are required to effect the cleavages in the presence of the higher concentration of monovalent salt (data not shown).

It is noteworthy that two of the cleavage sites (before A_{248} and U_{335}) coincide with sites in RNase P RNA which crosslink with tRNA containing a photoaffinity agent on its 5′ end (13). Four sites (before C_{123}, G_{229}, G_{259} and U_{294}) are near bases protected from modification with chemical probes when tRNA is bound to RNase P (T.LaGrandeur and N.R.Pace, unpublished data). Therefore, at least these particular cleavage sites are in the active portion of the ribozyme.

Pb^{2+} cleavage of RNase P RNAs from phylogenetically diverse organisms

In order to test the generality of the Pb^{2+} cleavage sites in the *E.coli* RNA, RNase P RNAs from *Chromatium vinosum* and *Bacillus subtilis* were also examined. The results are summarized in Figure 1. The cleavage sites exhibited by the *C.vinosum* RNase P RNA (data not shown) were practically identical to those of the *E.coli* RNA, consistent with the relatively close phylogenetic relationship of these organisms and the structural and kinetic similarities of the RNAs (14,16 see Fig. 1). The cleavage pattern exhibited by the *B.subtilis* RNase P RNA (Fig. 2B) is distinct from that of the *E.coli* and *C.vinosum* RNAs, consistent with the striking differences in the secondary structures of the RNAs (Fig. 1). Despite the extensive differences in sequence and structure, however, most of the cleavage sites occur in homologous sequence regions in all three RNAs. The cleavage sites are independent of the local sequence. Pb^{2+}-induced cleavage within the collection of RNAs occurs between 13 of the 16 possible dinucleotide sequences (Fig. 1). Two of the cleavages occur at exactly homologous positions in all the RNAs; before A_{135} and U_{256} in the *B.subtilis* RNA, which correspond to C_{123} and G_{259} in the *E.coli*, and A_{104} and G_{236} in the *C.vinosum* RNAs. The identification of two sites of Pb^{2+} cleavage at exactly homologous positions in the RNAs of the three organisms studied suggests a particularly important role for these regions in the folding of the RNA and possibly in the catalytic mechanism, which requires divalent cations (1).

The cleavage site before A_{135} in the *B.subtilis* RNA consistently appears as one of the most efficient sites of cleavage, occurring at the lowest Pb^{2+} concentrations (Fig. 2B). The second identical cleavage site in all the RNAs, before U_{256} in

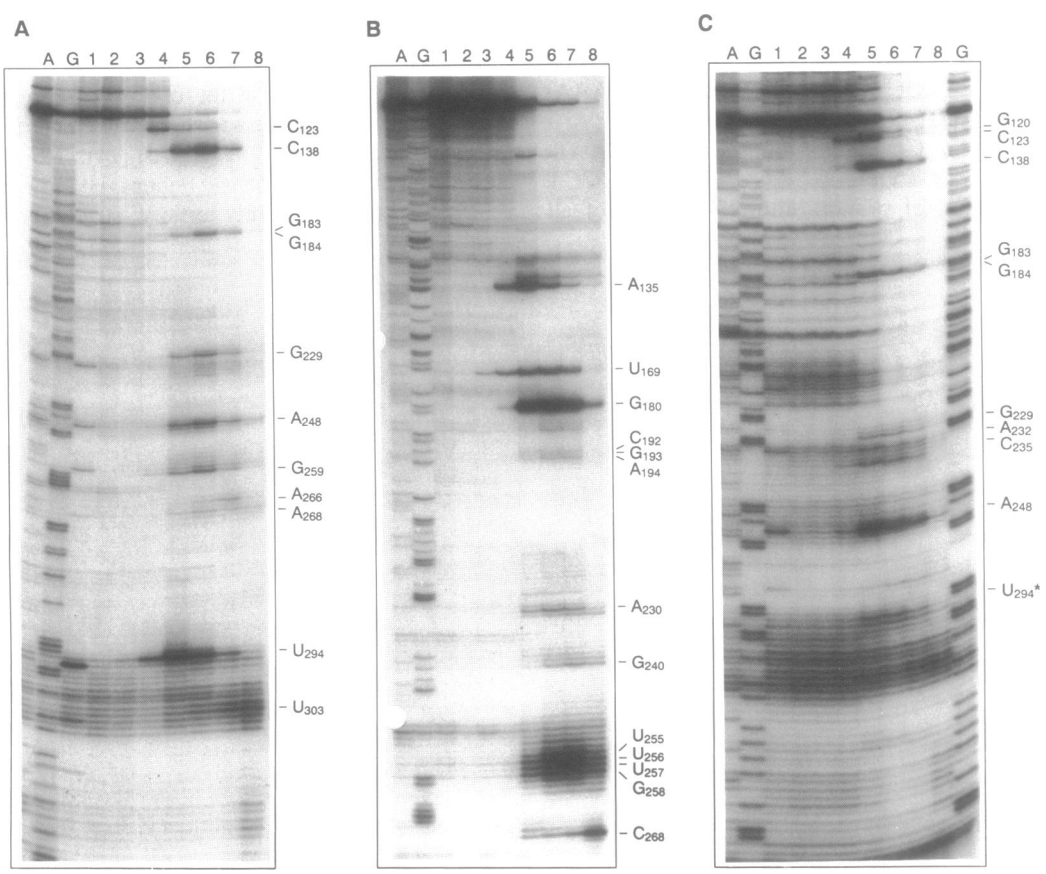

Figure 2. Primer extension analysis of *E.coli*, *B.subtilis*, and Δ4 RNase P RNA lead-catalyzed cleavage products. Pb^{2+} cleavage was performed as described in Materials and Methods. In all cases, $Pb(OAc)_2$ concentrations for the reactions in lanes 1−8 are 0, 0, 0.05, 0.1, 0.25, 0.5, 1, and 5 mM, respectively. Lanes A and G are dideoxynucleotide-terminated (ddA and ddG) sequencing reactions carried out on the respective unmodified RNase P RNAs. Primers used were 282R for *B.subtilis* and 352R for *E.coli* and Δ4 RNAs (Materials and Methods). Positions at which reverse transcription is halted due to cleavage of the RNA are indicated with the nucleotide identity and position in the RNase P RNA. (**A**) Primer extension of *E.coli* Pb^{2+}-catalyzed cleavage products. (**B**) Primer extension of *B.subtilis* Pb^{2+}-catalyzed cleavage products. (**C**) Primer extension of Δ4 Pb^{2+}-catalyzed cleavage products (* indicates numbering based on wild-type *E.coli* RNase P RNA).

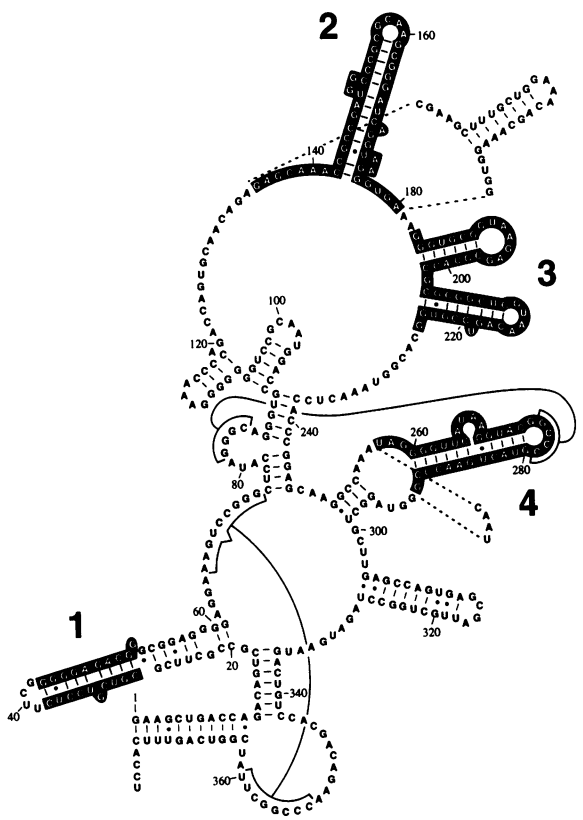

Figure 3. *E.coli* RNase P RNA deletion mutants. The regions that were deleted from the *E.coli* RNase P RNA are highlighted and numbered. In addition to the deletion of helix 1 in the Δ1 mutant, U25 was changed to a C in order to increase the theoretical stability of the shortened helix. In the case of Δ2Δ3 and Δ4, sequences from the RNase P RNA of the Gram-positive bacterium *B.megaterium* were substituted for the highlighted sequences to create the mutant RNAs. For Δ2Δ3, the deletion of helices 2 and 3 was accompanied by replacement of the helix 2 highlighted sequences with the sequence shown connected by the dashed lines. For Δ4 also, the sequence connected by the dashed lines replaced the highlighted sequences. These mutants were previously described and characterized in detail (5).

the *B.subtilis* RNA, is surprising considering the absence of a helix in the *Bacillus* RNA that would correspond to the helix formed by nucleotides 260−290 in the *E.coli* RNA (nucleotides 237−282 in *C.vinosum*). Since Pb^{2+}-cleavage seems to indicate the occurrence of tertiary structure, the extensive cleavage observed in this region of the *B.subtilis* RNA suggests that this helix loop is involved in tertiary interaction with another region of the RNA molecule. This is despite the lack of an identifiable long-range base-pairing interaction as occurs in the *E.coli* RNA (Fig. 1). It has been proposed that another (unidentified) structural element in the *Bacillus* RNA could independently fulfil the function of the helix composed of nucleotides 260−290 in the *E.coli* RNA (3).

Pb^{2+} cleavage is an effective assay for structural integrity of mutant RNAs

The cleavage patterns of three mutant *E.coli* RNase P RNAs were examined. These mutants, described and characterized extensively in a previous study (5), contain deletions of structural elements which are variably present in RNase P RNAs of other organisms (Fig. 3). In two cases, Δ2Δ3 and Δ4, sequences from the RNase P RNA of *Bacillus megaterium*, a close relative of *B.subtilis*, were substituted to create the mutant RNA. In Δ1 RNA, in addition to the deletion, U_{25} was changed to a C in order to increase the theoretical stability of the helix that was shortened by the mutation. Each of the mutants is catalytically active *in vitro*. However, the mutants Δ2Δ3 and Δ4 were suggested to have structural defects, as indicated by higher ionic strength requirement for catalysis (Δ2Δ3) or temperature-sensitivity (Δ4), relative to the native RNA (5).

Table I. Cleavage sites of *E.coli* RNase P RNA and deletion mutants

site*	*E.coli*	Δ1	Δ2Δ3	Δ4
C123	+	+	−	+
C138	+	+	−	+
G183	+	+	−	+
G184	+	+	−	+
G229	+	+	−	weak
A248	+	+	+	+
G259	+	+	weak	−
U294	+	+	+	weak
U335	+	+	+	+

*Sites are indicated according to position in *E.coli* RNase P RNA. Specific Pb^{2+} cleavage comparable to the extent observed with the native *E.coli* RNA is indicated by a plus sign. Minus signs indicate no specific cleavage.

The Pb^{2+}-catalyzed cleavage pattern of Δ1 RNA is almost identical to that seen for the native *E.coli* ribozyme (summarized in Table I). The nine major cleavage sites are the same; differences are observed only in minor cleavage sites. This result is consistent with previous characterizations of this RNA, indicating that the shortening of the mutated helix has little effect on the structure or kinetic capabilities of the enzyme (5). Considering the sensitivity of the Pb^{2+} cleavage assay to any perturbations in tertiary structure (7), this result shows the utility of the assay to detect native structure in mutant RNAs.

Pb^{2+} cleavage sites in Δ4 RNA differ extensively from those in the native *E.coli* RNA, indicating a significant structural disruption. Three of the nine major sites of cleavage are affected (Table I, Fig. 2C). At two sites, before G_{229} and U_{294}, cleavage is still observed, but at a reduced level; cleavage before G_{259} does not occur. The absence of cleavage at this site is striking because of the conservation of the cleavage between species (above). Even in the *B.subtilis* RNA, which naturally lacks helix 4, Pb^{2+}-catalyzed cleavage occurs in this region, indicating its involvement in tertiary structure. However, the majority of the Pb^{2+} cleavage sites are intact in the Δ4 mutant, with only the local disturbance in the helix 4 region (loss of cleavage before G_{259}). These results are consistent with the properties of this mutant: high K_M and temperature-sensitivity indicate loss of interactions stabilizing the structure (5), yet the lack of a particularly high ionic strength requirement for activity indicates that the structural disruption is local instead of global.

The cleavage sites exhibited by the mutant Δ2Δ3 RNA are the least similar among these mutants to cleavages which occur in the native ribozyme (Table I). Only three of the nine major sites of cleavage in the native RNA remain in the mutant, those occurring before A_{248}, U_{294} and U_{335}. Many new minor cleavage sites appear in this mutant RNA (data not shown), possibly indicating a loosening of its entire structure, leading to increased accessibility to random cleavage by Pb^{2+}. Both of the major phylogenetically conserved cleavage sites are lost in this mutant RNA, indicating severe disruption of tertiary structure.

A particularly high ionic strength requirement for catalysis by Δ2Δ3 RNA (5) is consistent with such extensive structural disruptions. High ionic strength is thought to enhance the activity of mutant RNase P RNAs by screening electrostatic repulsion (due to nucleotide phosphates) that otherwise would distort the structure of a mutationally destabilized RNA (4). The extensive disruption in the Pb^{2+} cleavage pattern of the Δ2Δ3 likely reflects the destabilization of the global tertiary structure.

DISCUSSION

Understanding the mechanism of the RNase P processing reaction will require the identification of structures that are important for catalysis. It is likely that tertiary structure, as scaffolded by secondary structural elements, plays the main role in determining the specificity of this enzyme–substrate interaction. Assessment of the integrity of tertiary structure is, therefore, an important consideration in mutational studies of RNase P. Until recently, the primary assay implemented in the study of mutants of RNase P was determination of catalytic activity, i.e., cleavage of labeled pre-tRNA. This assay is limited, however, since it does not distinguish between mutants that are specifically defective in binding or catalysis from those that are inactive because of distortion of the global structure. These results show that the Pb^{2+} cleavage pattern is an assay for integrity of global structure. Features that make the Pb^{2+} cleavage assay attractive are: 1) RNA structure is scored independently of catalytic ability; 2) the reaction is highly specific for the native structure; and 3) Pb^{2+} appears to show no preference for cleavage before or after a particular nucleotide. The mutant RNase P RNAs studied here exhibit Pb^{2+} cleavage patterns that are distinct from the native pattern and, consonant with other properties of the mutants (5), indicate destabilization of tertiary structure. The Pb^{2+} cleavage patterns can, therefore, be useful for detecting mutational perturbations of tertiary structure.

The occurrence of two cleavage sites at exactly homologous positions in three structurally distinct RNase P RNAs, from three different organisms, suggests a possible role of these cleaved regions in establishing a common tertiary structure in RNase P RNAs. Such conservation in structure might suggest that these regions are involved in some specific role of the RNA, for instance in substrate-binding or catalysis. RNase P requires divalent cations (optimally Mg^{2+} or Mn^{2+}) for catalysis (1, 16, 17), but not for the assumption of the proper structure for binding the substrate (16). It is possible that the sequence regions involved in forming the conserved Pb^{2+} cleavage sites also are involved in the specific binding of Mg^{2+} ions; three Mg^{2+} ions are required for the maximal rate of cleavage by RNase P (17). It is noteworthy that the regions involved in the two conserved Pb^{2+} cleavage sites, at C_{123} and G_{259} in the *E.coli* RNA, also were seen by Kazakov and Altman to undergo cleavage by Mg^{2+} at pH 9.5 (18), although the exact sites of Mg^{2+}-promoted cleavage were identified as 2–3 nucleotides removed from the Pb^{2+}-induced cleavages. Moreover, the conserved Pb^{2+}-promoted cleavages are not observed in the presence of >50mM Mg^{2+} (A.Hüttenhofer, unpublished results), consistent with the notion that Pb^{2+} ions can replace and cleave RNA at Mg^{2+}-binding sites. This is also in agreement with studies of $tRNA^{Phe}$, in which a Pb^{2+} ion that cleaves the D-loop has been found to displace a Mg^{2+} ion specifically bound nearby in the native structure (11, 19). More recently, Pb^{2+}-promoted cleavage has been observed at a putative Mg^{2+}-binding site in a group I intron (15). Therefore, the conserved Pb^{2+} cleavage sites in RNase P RNAs may identify sequence regions where catalytic Mg^{2+} ions are bound.

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