Probing RNA Structure by Lead Cleavage

This unit describes the probing of RNA structure using Pb(II) ions. The Pb(II) hydroxide species has a relatively low pK_a (~7.7), which allows it to extract the proton from a 2′-hydroxyl group. In RNA, abstraction of a 2′ proton increases the nucleophilicity of the 2′ oxygen, facilitating attack on an adjacent phosphodiester bond. This transesterification reaction breaks the 5′,3′ phosphodiester backbone of the RNA to generate 2′,3′ cyclic phosphate and 5′-hydroxyl products. Since the efficiency of 2′ hydroxyl proton abstraction is related to steric and chemical constraints on particular 2′ OH groups embedded in the RNA structure, the Pb^{2+} cleavage rate can be used to examine the structure of individual nucleotides within an RNA molecule. This method is a particularly sensitive probe of the tertiary structure of an RNA, provided that appropriate Pb^{2+} binding sites are created in the tertiary structure.

In order to detect the cleavage site and the cleavage rate by Pb^{2+}, RNAs are labeled with ^{32}P at either the 5′ or 3′ end (see Support Protocol 1 and 2). The labeled RNA is then subjected to Pb^{2+} cleavage (see Basic Protocol). The reaction mixture is analyzed on denaturing polyacrylamide gels containing 8 M urea. Partial alkaline hydrolysis reaction products and nuclease T1 digests of the same labeled sample are run alongside the cleavage reaction products to accurately locate the cleavage site (see Support Protocol 4). For optimal results, RNA should be renatured after labeling to ensure conformational homogeneity (see Support Protocol 3 to determine proper renaturation conditions). Reverse transcription runoff (*UNIT 6.1*) can also be applied to detect cleavages in larger RNAs.

CAUTION: All personnel should be trained in working with radioactivity.

NOTE: Extremely careful precautions should be taken in doing RNA work. Wear disposable gloves at all times to prevent nuclease contamination from your hands. Use the highest-quality water available and autoclave water prior to all solution preparation. It is not usually necessary to treat the water with diethyl pyrocarbonate (DEPC) if all possible precautions are taken to avoid nuclease contamination.

NOTE: Use silanized (low-adhesion) tubes for the cleavage reaction to minimize adsorption of RNA to the tube wall. This is especially critical if the reaction products are to be quantitated.

PROBING THE RNA STRUCTURE BY Pb^{2+} CLEAVAGE

BASIC PROTOCOL

If one is to obtain interpretable and reproducible results, there is much more to the Pb^{2+} cleavage reaction than simply mixing all the components and taking aliquots over time. Since RNA can have different conformations depending on a variety of external factors, Pb^{2+} cleavage under one set of conditions may not accurately reflect the biologically relevant structures one seeks to analyze. Therefore, to ensure correct interpretation, optimization of reaction conditions should be performed (see Critical Parameters) prior to conducting the final experiment. This section describes the basic protocol for a standard Pb^{2+} cleavage reaction.

Materials

5′ or 3′ ^{32}P-labeled RNA in water (see Support Protocols 1 and 2)
0.3 M buffer (see Critical Parameters)
Urea loading buffer (*APPENDIX 2A*)

Contributed by Tao Pan
Current Protocols in Nucleic Acid Chemistry (2000) 6.3.1-6.3.9

$Pb(OAc)_2$ stock solution prepared at 10× desired final concentration in reaction (see recipe for 10 mM solution)

Heating block
3-mm filter paper (Whatman)
Phosphorimager with appropriate software and phosphor screens

Additional reagents and equipment for RNA renaturation (see Support Protocol 3), partial alkaline hydrolysis and T1 nuclease digestion (see Support Protocol 4), and denaturing polyacrylamide gel electrophoresis (see *APPENDIX 3B*)

Cleave RNA

1. If necessary, renature RNA sample (see Support Protocol 3 to determine proper renaturation conditions).

   Renaturation is advisable if the RNA or source cells have been subjected to any potentially denaturing conditions; see Support Protocol 3 for details.

   Renaturation, which refers to folding, is different for every RNA. To obtain proper tertiary structure in vitro, [RNA], [Mg^{2+}], and temperature will need to be varied. The exact amount of RNA to use should be determined empirically, as should the other parameters, when experimental conditions are optimized; however, enough RNA to give a concentration of 0.1 to 1 μM in 50 μL is a good starting point. See Critical Parameters for further discussion.

2. Dilute RNA to 50 μL with water and enough 0.3 M buffer to provide a 20 mM final buffer concentration.

   Renaturation should leave the RNA appropriately diluted.

3. Take a 4.5-μL aliquot of the RNA/buffer mix and mix with an equal volume of denaturing-gel loading buffer.

   This is the time-zero RNA sample.

4. Add 5 μL of 10× $Pb(OAc)_2$ solution to the remaining RNA to initiate cleavage.

   $Pb(OAc)_2$ concentration should be optimized with other experimental conditions; see Critical Parameters for discussion.

5. Incubate at 20° to 40°C.

   See Critical Parameters for discussion of reaction temperature.

6. Remove samples at selected time points: e.g., 5-μL aliquots at 0.5, 1, 2, 4, 8, 15, 30, and 60 min. To stop the reaction, mix an equal volume of urea loading buffer with each aliquot as it is taken.

7. Perform partial alkaline hydrolysis and T1 nuclease digestion of the same RNA (see Support Protocol 4).

8. Analyze the Pb^{2+} reaction mixture on a high-resolution denaturing polyacrylamide gel containing 8 M urea. Include samples of partial alkaline hydrolysis and T1 nuclease digestion reactions on the same gel.

   The author uses a 15% gel for a 76-mer RNA.

9. Compare the cleavage product band with the partial alkaline hydrolysis and T1 nuclease reaction bands to determine the cleavage site.

Analyze data

10. Transfer the sequencing gel to a piece of 3-mm Whatman filter paper, cover with Saran Wrap, and dry.

11. Expose the gel on a phosphorimager plate at ambient temperature.

    Exposing the gel in a freezer does not improve the result although exposure at 4°C can lead to sharper bands.

12. Scan the phosphor plate and quantitate the full-length RNA using volume count, cpm substrate (S), and any reaction products of interest, cpm product (P):

    $\%S = cpm(S)/[cpm(S) + cpm(P)]$

13. Plot ln(%S) versus reaction time and fit with a linear equation. The slope of the curve is the observed cleavage rate (k_{obs}).

    The cleavage reaction may be biphasic when all time points are plotted on the same graph. This observation is often due to the heterogeneity of RNA conformation (see Support Protocol 3). In this case, use only the early time points, until ~20% to 50% of the RNA are cleaved.

14. Compare k_{obs} values for reactions under different conditions for optimal interpretation (see Commentary).

5′ ^{32}P-LABELING OF RNA INCLUDING DEPHOSPHORYLATION

SUPPORT PROTOCOL 1

RNA can be ^{32}P labeled at the 5′ end with [γ-^{32}P]ATP and T4 polynucleotide kinase. Chemically synthesized oligoribonucleotides generally contain a free 5′ hydroxyl group, which can be labeled directly. RNAs made by in vitro transcription generally contain a 5′ triphosphate group that has to be removed with alkaline phosphatase prior to the 5′ labeling reaction.

Label enough RNA for both the cleavage reaction (Basic Protocol) and the preparation of T1 and partial alkaline hydrolysis standards for electrophoresis (Support Protocol 4).

Materials

RNA sample in water
1 M Tris·Cl, pH 8.0 (*APPENDIX 2A*)
1 U/μL calf-intestine alkaline phosphatase
Soaking buffer: 50 mM potassium acetate (*APPENDIX 2A*)/200 mM KCl, pH ~7
1:1 (v/v) phenol/chloroform (*APPENDIX 2A*)
Ethanol
10× T4 polynucleotide kinase buffer (see recipe)
[γ-^{32}P]ATP (use highest activity available)
T4 polynucleotide kinase
Urea loading buffer (see *APPENDIX 2A*)
Polyacrylamide gel containing 8 M urea
Sodium acetate, pH 5.2 (*APPENDIX 2A*)

Micropipettor
Speedvac evaporator (e.g., Savant)
X-ray film
RNase-free surgical blade

Additional reagents and equipment for denaturing polyacrylamide gel electrophoresis (see *APPENDIX 3B*)

NOTE: If phosphatase treatment is not needed, proceed directly to step 7.

NOTE: All microcentrifugations are performed at full speed.

Remove 5′ triphosphate group

1. To 10 to 50 pmol RNA dissolved in water, add 1.5 µL of 1 M Tris·Cl, pH 8.0, and water to 29 µL.
2. Add 1 µL of 1 U/µL calf-intestine alkaline phosphatase. Incubate 30 min at 37°C.

Purify phosphatase-cleaved RNA

3. Add 20 µL soaking buffer; then extract alkaline phosphatase with 50 µL of 1:1 (v/v) phenol/$CHCl_3$, mix, and let stand 1 min at room temperature. Microcentrifuge 1 min and collect the top layer into a fresh tube.
4. Add 140 µL ethanol and incubate 20 min at −20°C.
5. Microcentrifuge 20 min at 4°C.

   Align tubes in the microcentrifuge so that the hinges of the tubes are facing the center of the rotor. The pellet will adhere to the tube on the side opposite the hinge.

6. Carefully pipet out the liquid. Dry the RNA pellet in a Speedvac evaporator.

   The pellet may not be visible to the naked eye.

Label RNA at 5′ end with ^{32}P

7. To the RNA add in following order (10 µL final volume):

   1 µL 10× T4 polynucleotide kinase buffer
   H_2O
   1 to 1.5 mol equiv [γ-^{32}P]ATP
   3 to 5 U T4 polynucleotide kinase.

8. Incubate 30 min at 37°C.

Purify 5′-labeled RNA

9. Add 10 µL denaturing-gel loading buffer and purify on a denaturing polyacrylamide gel containing 7 M urea.

   The author uses a 10% gel for a 76-nucleotide RNA; see APPENDIX 3B.

10. Locate the radioactive band by exposing the gel briefly on X-ray film. Use a fluorescent ruler for orientation.

    If the RNA did not become labeled with ^{32}P, repeat using a new batch of alkaline phosphatase in step 2.

11. Develop the film and make a template that will be used to locate the band containing your RNA.
12. Lay the template on the gel, using the rulers as a guide. Cut out the RNA band with an RNase-free surgical blade. Elute RNA in the soaking buffer for at least 1 hr at ambient temperature.
13. Add sodium acetate, pH 5.2, to 0.25 M (final) and 2.5 vol ethanol to precipitate the RNA. Mix and incubate at least 5 min on crushed dry ice or 30 min at –20°C. Microcentrifuge to remove supernatant. Dry the pellet and redissolve in 10 µL water.

3′ ^{32}P LABELING OF RNA USING T4 RNA LIGASE AND [^{32}P]pCp

RNA can be labeled at the 3′ end with 5′ [^{32}P]pCp and T4 RNA ligase. Steps 1 to 3 describe the synthesis of 5′ [^{32}P]pCp. For additional detail on the reaction, see 3′ RNA labeling in *UNIT 6.1*. In general, the RNA should be labeled such that at least 20,000 cpm will be loaded per lane.

Be sure to label enough RNA to have extra for the preparation of the sizing standards for electrophoresis (Support Protocol 4) in addition to what will be needed for the Basic Protocol. This procedure is designed for labeling 20 to 100 pmol RNA.

Materials

10× T4 polynucleotide kinase buffer (see recipe)
[γ-^{32}P]ATP
3′ cytosine monophosphate (3′ Cp)
T4 polynucleotide kinase
Dimethyl sulfoxide (DMSO)
100 µM nonradioactive ATP
T4 RNA ligase
RNA sample in water
Soaking buffer: 50 mM potassium acetate (*APPENDIX 2A*)/200 mM KCl, pH ~7
Urea loading buffer (*APPENDIX 2A*)

Additional reagents and equipment for purification of ligated RNA (see Support Protocol 1)

NOTE: If commercial 5′ [^{32}P]pCp (e.g., New England Nuclear) is used, proceed directly to step 4. Depending on the concentration supplied, use a quantity sufficient to provide the same molar ratio as achieved with [γ-^{32}P]ATP.

1. Mix in the following order (20 µl final volume):

   2 µL 10× T4 polynucleotide kinase buffer
   H_2O
   1 mol equiv [γ-^{32}P]ATP to 3′ Cp (~1 to 2 mol equiv RNA to be ligated)
   3 to 5 U T4 polynucleotide kinase.

2. Incubate 30 min at 37°C.

3. Heat at 75°C for 3 min to inactivate kinase, then immediately place on ice for at least 3 min.

4. Add the following (30 µL final volume including RNA added in step 5):

   3 µL 10× T4 polynucleotide kinase buffer (1× final)
   4.5 µL DMSO (15% final)
   1.8 µL 100 µM nonradioactive ATP (6 µM final)
   0.7 U/µL T4 RNA ligase

5. Add ≤3 µL of RNA sample in water and incubate 4 to 19 hr at 16°C.

   If the RNA sample is too dilute, concentrate the RNA first in a Speedvac.

6. Add 30 µL urea loading buffer. Purify the ligated RNA as described for 5′-labeled molecules (see Support Protocol 1, steps 9 to 12).

OPTIMIZATION OF RNA RENATURATION

It is generally not difficult to observe Pb^{2+} cleavage of an RNA; however, caution is necessary for proper interpretation. RNAs are capable of forming multiple conformations that may be kinetically trapped (Uhlenbeck, 1995). This is particularly true for RNAs that are purified from denaturing polyacrylamide gels. For those RNAs, a renaturation step is mandatory to ensure conformational homogeneity. For RNAs that are isolated from cell extracts and have not gone through any steps that are likely to cause denaturation, renaturation is probably not necessary. However, if the cell extract has been subjected to the presence of millimolar amounts of EDTA, high temperature (>60°C), or phenol extraction, the RNA may have been denatured and renaturation should be attempted.

The following procedure should be used to determine optimal conditions for renaturing a particular RNA species.

Materials

RNA sample in water
MES, MOPS, or HEPES buffer
$MgCl_2$ stock solution, prepared at 10× desired final concentration in reaction (*APPENDIX 2A* for 1 M stock solution)
Heating block
Additional materials for nondenaturing polyacrylamide gel electrophoresis (see e.g., *CPMB UNIT 2.7*)

1. Mix RNA with buffer and water to obtain a final RNA concentration of ~0.1 to 1 μM. Prepare triplicate tubes.
2. Heat 2 min at 85° to 90°C using a heating block.

   This step should denature any alternate secondary-structure conformers in the absence of divalent ions.

3. Take the sample out of the heating block. Either leave tube at room temperature for 3 min and then microcentrifuge briefly, *or* turn the heat block off and let the sample slowly cool to room temperature.

   The best method for cooling the sample will depend on the RNA and has to be determined empirically. In general, it is best to start with the slow cooling.

4. Add 10× $MgCl_2$ solution to the desired Mg^{2+} concentration. Incubate tubes at three different temperatures (recommended: ambient, 37°C, and 50°C) for 10 min.

   To prevent nonspecific Mg^{2+}-induced hydrolysis, do not heat the RNA to above ~60°C in the presence of Mg^{2+}.

5. Add other components, if required (e.g., 0.1 M KCl, 1 mM spermine). Incubate again at 37°C for 5 min.
6. Analyze all samples on a nondenaturing polyacrylamide gel (see, e.g., *CPMB UNIT 2.7*).
7. Assess the gel to determine which conditions produced the best renaturation, and use these when renaturing samples of this RNA species.

   Properly renatured RNA should show the following general properties when run on a nondenaturing gel (although these may differ among individual RNAs): (1) a single band on the gel, suggesting conformational homogeneity, and (2) fast migration relative to RNA in the absence of Mg^{2+}, suggesting compactness. Most, if not all, RNAs with tertiary structure require the presence of a divalent ion (e.g., Mg^{2+}) to renature correctly.

PARTIAL ALKALINE HYDROLYSIS AND NUCLEASE T1 DIGESTION

Aliquots of the end-labeled RNA are used to generate markers to size and analyze the Pb cleavage products. A ladder of truncated RNAs is generated by alkaline hydrolysis, while the T1 nuclease cleaves the RNA at guanosines.

Materials

5′- or 3′-end-labeled RNA (≥200,000 cpm; see Support Protocols 1 and 2)
1 mg/mL *E. coli* tRNA mixture (Sigma), dissolved in water
5× alkaline hydrolysis (AH) buffer: 5 mM glycine/2 mM $MgSO_4$, pH 9.5
1 U/µL ribonuclease T1, diluted in water from a 100 U/µl stock (store up to ~1 month at −20°C)
Urea loading buffer (*APPENDIX 2A*)
100°C water bath

1. To 0.2 to 1.0 µl 5′- or 3′-end-labeled RNA, add water to 3 µL. Prepare two duplicate tubes.

   The exact amount of RNA will depend on the level of radioactivity.

2. Add 1 µL *E. coli* tRNA to each tube.

   This ensures that all reactions are carried out at a similar ratio of RNA to nuclease T1.

3. To one tube (alkaline hydrolysis condition), add 1 µL of 5× AH buffer, then boil 1 min, in a 100°C water bath.

4. To the second tube (T1 digestion condition), add 1 µL nuclease T1 (1 U), then incubate 2 min at 65°C.

5. Quick-cool 3 min on ice.

   CAUTION: *Do not keep the T1 reaction mixture for >30 min, as T1 continues to cut RNA, even on ice.*

6. Microcentrifuge briefly.

7. Add 5 µL urea loading buffer to each tube.

   The samples are now ready for use as size standards for comparison with the Pb^{2+} cleavage products.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps; prepare all buffers with autoclaved water and then reautoclave. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

$Pb(OAc)_2$, 10 mM

Dissolve solid $Pb(OAc)_2$ in autoclaved water. Filter through 0.22-µm filters into sterile plastic tubes. Divide into aliquots and store at −70°C. Thaw and use one aliquot at a time; thawed aliquots can be stored at −20°C and used for ~1 week.

T4 polynucleotide kinase buffer, 10×

0.5 M Tris·Cl, pH 7.6 (*APPENDIX 2A*)
100 mM $MgCl_2$
100 mM 2-mercaptoethanol
Store up to ~1 year at –20°C

COMMENTARY

Background Information

The basic mechanism whereby Pb^{2+} cleaves RNA is proton abstraction by the polyhydrated $Pb(OH)^+$ species. Because of its low pKa (~7.7), $Pb(OH)^+$ is present in relatively high proportions at neutral pH. Compared to other divalent metal ions with higher pK_a (e.g., $Mg(H_2O)_5(OH)^+$ with a pK_a of ~11.4), $Pb(OH)^+$ base induces cleavage of the RNA backbone much more frequently to result in faster "background rates," defined as the cleavage rate of an RNA of any sequence independent of secondary or tertiary structure. For example, background rates have been measured at 50 μM $Pb(OAc)_2$, 10 mM $MgCl_2$, pH 7.0, 25°C to be ~0.003 min^{-1} (half-life ~4 hr; Pan and Uhlenbeck, 1992) and at 10 mM $MgCl_2$, pH 7.5, 25°C to be ~1×10^{-6} min^{-1} (half-life ~11,000 hr; Hertel et al., 1994). Thus, the background rate for Pb^{2+} cleavage is within the time scale for an impatient experimentalist, and Pb^{2+} cleavage can be easily carried out in reasonable amount of time. It should be emphasized, however, that due to the peculiar properties of hydrated $Pb(OH)^+$, the background rate does not necessarily increase linearly with total Pb^{2+} concentration (Kragten, 1978; Pan et al., 1994).

The actual observed Pb^{2+} cleavage rate (k_{obs}) is of course dependent on the RNA structure. The helical regions in RNA do not have the appropriate geometry for an in-line attack of the 2′ oxygen on its adjacent phosphate. Therefore, cleavage rates for nucleotide residues within an RNA helix are slower than the background rate. The completely unstructured regions in an RNA should be cleaved at the background rate. Hence, Pb^{2+} cleavage is a useful means for differentiating double-stranded from single-stranded regions in RNA. On the other hand, the single-stranded regions in RNA often have some kind of structure that can either enhance or inhibit the cleavage mechanism. The observed cleavage rates for nonhelical regions are often uneven. Nevertheless, without a specific Pb^{2+} binding site on the RNA (see below), cleavage of nonhelical regions rarely exceeds 10-fold the background rate.

Pb^{2+} ion(s) bound at well-defined sites in an RNA can significantly increase the cleavage rate at specific sites. These so-called "specific cleavage" reactions can have cleavage rates >1000-fold above the background rate (Pan and Uhlenbeck, 1992). Cleavage of yeast $tRNA^{Phe}$ is the most extensively studied case of a specific cleavage reaction by Pb^{2+}. Three Pb^{2+} binding sites have been found in the crystal structure of this tRNA, only one of which, Pb(1), results in specific RNA cleavage (Brown et al., 1985). Pb(1) is directly coordinated to nucleotides U59 and C60 in the TψC loop and cleaves the phosphodiester between U17 and G18 in the D loop. Site-directed mutagenesis of yeast $tRNA^{Phe}$ shows that the cleavage rate is sensitive to subtle changes in the tertiary structure of tRNA (Behlen et al., 1990). The Pb^{2+} cleavage rates are well-suited to assessment of structural changes in the tRNA tertiary fold upon mutations or site-specific modifications.

Critical Parameters

The three essential components of the cleavage reaction, RNA, Pb^{2+}, and Mg^{2+}, can act cooperatively to influence the cleavage rate. As a polyanion, RNA can bind divalent ions nonspecifically to decrease the concentration of free divalent ions in solution, so high concentrations of RNA should be avoided. On the other hand, nonspecific adsorption of RNA on the wall of a test tube can cause inaccurate measurement of cleavage rates, and this is exacerbated by low RNA concentration (the use of silanized tubes alleviates, but does not eliminate, this problem). Therefore, the RNA concentration in the reaction should be kept at ~20 to 200 μM in phosphate (e.g., yeast $tRNA^{Phe}$ = 76 phosphates). Use of >5 mM Pb^{2+} is not recommended since higher concentrations generate insoluble polyhydroxides that can coaggregate RNA, and aggregated RNA may be cleaved differently than RNA that is free in solution. How much Pb^{2+} to use in the reaction will depend on the concentration of Mg^{2+}, which can compete with Pb^{2+} binding to RNA. The Pb^{2+}/Mg^{2+} ratio is especially important for specific Pb^{2+} cleavage reactions since Mg^{2+} may bind at the same site or at an overlapping site to exclude simultaneous binding of Pb^{2+}. To maintain the RNA structure, Mg^{2+} concentration should be kept at 1 to 100 mM. A 1:50 $[Pb^{2+}]/[Mg^{2+}]$ ratio may be a good starting point for experimental optimization.

pH and temperature of the reaction also play a role in producing optimal results. Since the cleavage rate is often proportional to $Pb(OH)^+$ concentration, the reaction rate can be log-linearly dependent with pH below the pK_a of $Pb(OH)^+$. At pH above ~7.5, Pb^{2+} is more prone to form polyhydroxide aggregates. Therefore, Pb^{2+} cleavage should be carried out between

pH 6.0 and 7.7 (neutral pH is a good compromise). For the 0.3 M buffer, use MES, MOPS, or HEPES buffers prepared at an appropriate pH in autoclaved water, divided into aliquots, and reautoclaved. Inorganic buffers such as phosphates should not be used, since they may form insoluble salts with Pb^{2+}. Higher temperature accelerates the cleavage rate but may destabilize the RNA structure to result in unintended cleavage sites. On the other hand, kinetically trapped alternate conformers of RNA are more stable at low temperature. Therefore, the reaction temperature should be kept between 20° and 40°C.

Interpretation of cleavage results should be based only on primary cleavages, defined as the first cleavage event within the full-length molecule. It is possible, or even likely, that cleaving the backbone at one site can result in a conformational change in RNA to allow it be cleaved at other sites. These secondary cleavage sites are characterized by an initial lag in the kinetic analysis of the cleavage product. Therefore, it is crucial to perform time courses to ensure that interpretation is not based on secondary cleavage reactions.

Anticipated Results

The cleavage rates at individual sites can be used to deduce information on the structure of RNA and on structural changes resulting from mutations or other modifications. Cleavage of residues is generally faster in nonhelical regions than in helical regions, so cleavage rates can reflect the involvement of particular residues in the secondary structure. Cleavage at specific sites can be sensitive to structural changes. Based on Pb^{2+} cleavage alone, however, the precise molecular nature of such structural changes is difficult to understand; it requires further investigation by other methods. Finally, protein binding to an RNA can sterically hinder the access of the reaction site. Therefore, it should be possible to deduce some kind of "footprint" of a protein binding site by Pb^{2+} cleavage. Protein binding may also change the conformation of the RNA in the complex to enhance cleavage at other sites. The enhanced cleavage rates at such sites may be an excellent indication of conformational changes of RNA upon protein binding.

Time Considerations

All procedures in this unit are simple and can be completed in 2 days. Day 1 involves ^{32}P labeling and gel purification (see Support Protocols 1 and 2). Day 2 involves Pb^{2+} cleavage and gel analysis (see Basic Protocol). An extra day is needed for optimizing renaturation conditions (see Support Protocol 3).

Literature Cited

Behlen, L.S., Sampson, J.R., DiRenzo, A.B., and Uhlenbeck, C. 1990. Lead-catalyzed cleavage of yeast $tRNA^{Phe}$ mutant. *Biochemistry* 29:2515-2523.

Brown, R., Dewan, J., and Klug, A. 1985. Crystallographic and biochemical investigation of the lead(II)-catalyzed hydrolysis of yeast phenylalanine tRNA. *Biochemistry* 24:4785-4801.

Hertel, K.J., Herschlag, D., and Uhlenbeck, O.C. 1994. A kinetic and thermodynamic framework for the hammerhead ribozyme reaction. *Biochemistry* 33:3374-3385.

Kragten, J. 1978. Atlas of Metal-Ligand Equilibria in Aqueous Solution. Halsted Press, Chichester, UK.

Pan, T. and Uhlenbeck, O.C. 1992. A small metalloribozyme with a two-step mechanism. *Nature* 358:560-563.

Pan, T., Dichtl, B., and Uhlenbeck, O.C. 1994. Properties of an in vitro selected Pb^{2+} cleavage motif. *Biochemistry* 33:9561-9565.

Uhlenbeck, O.C. 1995. Keeping RNA happy. *RNA* 1:4-6.

Contributed by Tao Pan
University of Chicago
Chicago, Illinois