Chapter 9

Assessing Intermolecular RNA:RNA Interactions Within a Ribonucleoprotein Complex Using Heavy Metal Cleavage Mapping

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Abstract

Heavy metal cleavage mapping analysis of both assembling and fully mature ribonucleoprotein (RNP) complexes are informative techniques for assessing the intermolecular base pairing between small non-coding RNAs and their interacting target RNAs. Lead cleavage of the RNA in partially or fully assembled RNPs in the absence or presence of the interacting RNA can determine both the accessibility of the base pairing sequence within the RNP itself as well as its interaction with the target RNA. In this chapter, we detail how this technique was used to map the intermolecular RNA:RNA base pairing of a box C/D RNA with its target RNA within the assembling archaeal RNP complex.

Key words Intermolecular RNA:RNA base pairing, Lead cleavage mapping, RNP assembly, Gel electrophoresis, Non-coding RNA

1 Introduction

Intermolecular base pairing between small non-coding RNAs and other interacting or target RNAs is often a critical feature for small non-coding RNA function. Among the many examples are the base pairing of splicing small nuclear RNAs to pre-messenger RNA splice sites for splicing, microRNAs pairing with specific messenger RNAs to regulate translation or post-transcriptional stability, and the base pairing of small nucleolar RNAs with ribosomal RNAs to guide nucleotide modification [1–3]. Characteristically, these small non-coding RNAs exist as ribonucleoprotein complexes bound with a set of core proteins [1–3]. Nevertheless, the presentation of the base pairing sequence within the RNP complex is essential for engaging the target RNA and for small RNA function. Therefore, assessing exposure of this complementary sequence for intermolecular base pairing in an often dynamic RNP complex is frequently an important question for the investigation of small non-coding RNA/RNP structure and function (*see* Fig. 1).

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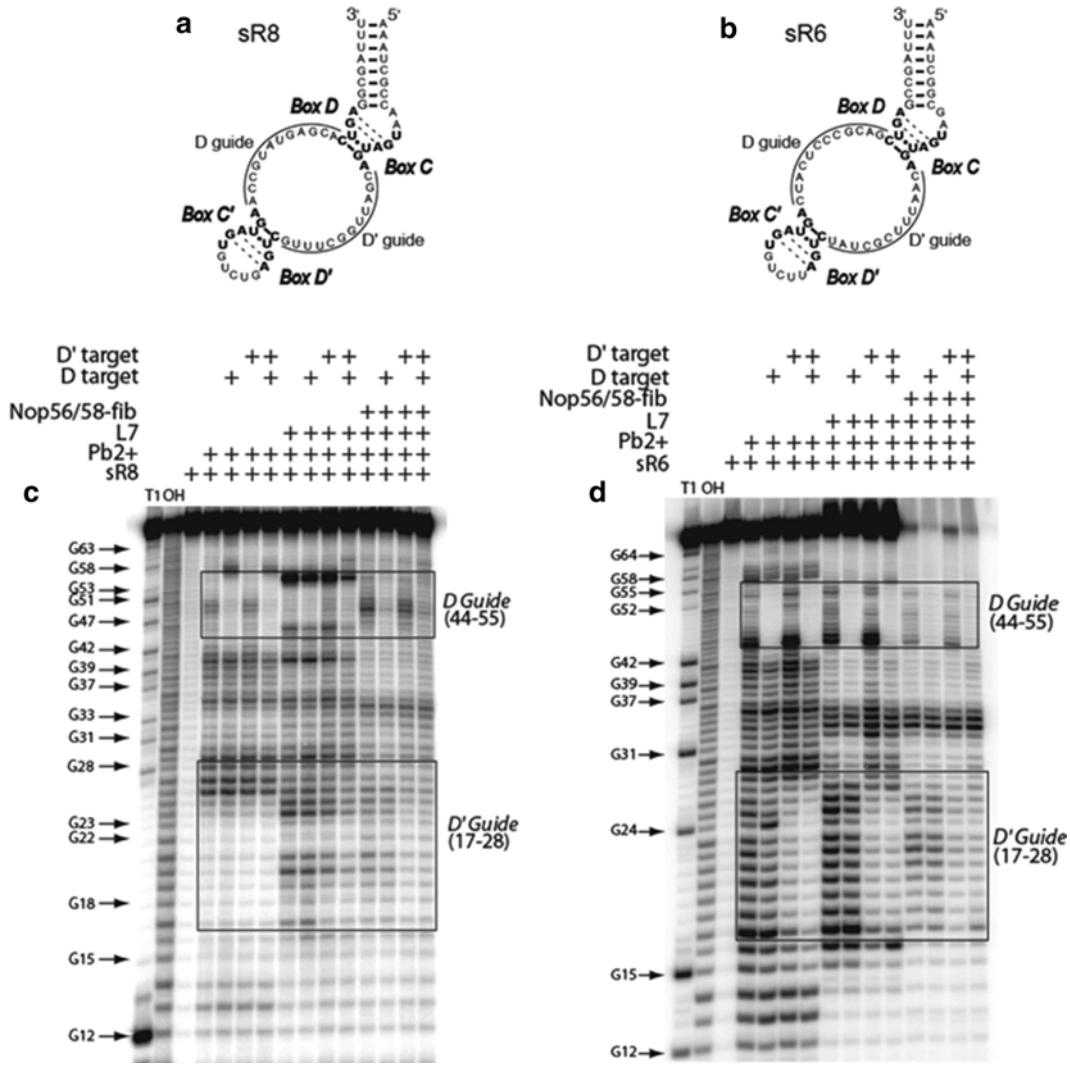

Fig. 1 Pb^{2+} cleavage mapping of box C/D sRNA guide sequences and their intermolecular base pairing with target RNAs. (Panels **a** and **b**) Schematic presentation of *M. jannaschii* sR8 (panel **a**) and sR6 (panel **b**) box C/D sRNAs. Boxes C/D and C′/D′ are indicated in *bold* with the respective D and D′ guide sequences designated with overlaid *bold lines*. (Panels **c** and **d**) Pb^{2+}cleavage mapping of the sR8 (panel **c**) and sR6 (panel **d**) box C/D sRNA D and D′ guide sequences and their intermolecular base pairing with their respective target RNAs during box C/D sRNP assembly. The sR8 and sR6 box C/D sRNPs were assembled in vitro with the sequential addition of the L7Ae, Nop56/58, and fibrillarin core proteins (indicated above each panel). At individual steps in sRNP assembly, the accessibility of the respective D and D′ guide sequences was assessed using Pb^{2+}cleavage mapping (indicated above each panel). Similarly, the ability of the guide sequences to base pair with complementary target RNAs and thus protect the guide sequences from Pb^{2+} cleavage was also assessed. RNase T1 cleavage (G nucleotides) and alkaline hydrolysis sequencing ladders for each sRNA are indicated above the respective gel *lanes*. The respective D and D′ guide sequences for each sRNA are enclosed in *boxes*. The sR8 D but not D′ guide sequence is accessible for both Pb^{2+} cleavage and target RNA binding during sRNP assembly. However, both sR8 guide sequences are accessible upon complete sRNP assembly. In contrast, both D and D′ guide sequences of sR6 were accessible to both Pb^{2+} cleavage and target RNA binding throughout sRNP assembly. (Figure reproduced from [4] with permission from Elsevier)

Traditional approaches to mapping folded RNA structure have included cleaving single-stranded regions with nucleases or heavy metals as well as modifying exposed sequences with nucleotide modification reagents [4]. Similarly, protein binding sites on a given RNA sequence have also been mapped using these same techniques where protein binding stabilizes and protects the RNA and limits accessibility of enzymes, heavy metals or modification reagents [5]. Importantly, these same approaches can also be used to assess intermolecular base pairing interactions between multiple RNAs, both during RNP assembly and in mature RNP complexes.

In this chapter, we describe how we have utilized lead cleavage of assembling box C/D small RNP (sRNP) complexes to assess the accessibility of the guide sequence for target RNA base pairing [4]. Our results demonstrate that the sR8 small RNA (sRNA) undergoes RNA remodeling during RNP assembly, ultimately exposing the guide sequences upon complete RNP assembly. In contrast, the guide sequences of a related sRNA, sR6, are exposed throughout RNP assembly. These observations helped us conclude that sRNP assembly is dynamic and requires presentation of accessible guide sequences for base pairing to target RNAs. They also illustrate the power of this simple experimental approach in characterizing the assembly of dynamic RNP complexes and assessing intermolecular RNA:RNA interactions.

2 Materials

2.1 Chemicals and Reagents

1. Purified, 5′ end radiolabeled box C/D RNA.
2. Purified box C/D RNP core proteins (for RNP assembly).
3. RNase-free distilled/deionized water (ddH_2O).
4. 10× Assembly Buffer: 0.2 M HEPES, pH 7.0, 1.5 M NaCl, 15 mM $MgCl_2$, 50 % glycerol (w/v).
5. Yeast transfer RNA (tRNA) at 10 mg/mL in ddH_2O.
6. Lead acetate, molecular biology grade.
7. T1 RNase (Ambion).
8. 10× Alkaline Hydrolysis Buffer: 0.1 M $NaHCO_3$, pH 10.0, 10 mM EDTA.
9. Lithium perchlorate ($LiClO_4$).
10. Acetone.
11. 40 % acrylamide, 19:1 (acrylamide:bisacrylamide cross-linking) stock solution.
12. 10× TBE: 0.89 M Tris base, 0.89 M boric acid, 20 mM EDTA.
13. Urea (molecular biology grade).
14. 10 % ammonium persulfate (APS), prepared fresh.

15. *N*,*N*,*N*′,*N*′-Tetramethyl-ethylenediamine (TEMED).
16. Glycerol (molecular biology grade).
17. Loading buffer: 90 % formamide, 1× TBE, 10 mM EDTA, 0.25 mg/mL bromophenol blue, 0.25 mg/mL xylene cyanol.
18. Acetic acid.
19. Ethanol.

2.2 Equipment and Supplies

1. Temperature-controlled heating block.
2. Automatic pipettor and pipettes.
3. Power supply (1,000+ Volts, constant power setting).
4. Sequencing gel equipment (C.B.S. Scientific): gel running apparatus, glass plates (42 × 36 × 0.5 cm and 40 × 36 × 0.5 cm), gel spacers (0.2 mm × 42 cm), 34-well comb (0.2 mm × 33 cm).
5. Large spring clips.
6. 0.2 mm flat gel loading tips.
7. Hoefer Wonder Wedge®.
8. Clear plastic wrap (Saran™ wrap or Glad™ wrap).
9. 3MM Whatman filter paper.
10. Temperature-controlled vacuum gel drier.
11. Phosphorimager and cassette or dark room, x-ray film and developer.

3 Methods

3.1 General Methods

We have previously described methods for practicing RNase-free technique, phenol–chloroform extraction of RNA solutions, precipitation of RNA, 5′-end radiolabeling of RNA, purification of radiolabeled RNA and in vitro transcription of box C/D RNAs [4, 5]. We refer to these protocols, which will be helpful for preparing and performing the assays described below.

3.2 Lead Cleavage for Determining RNA:RNA Base Pairing Interactions

For mapping experiments, radiolabeled RNA that has been gel-purified is preferred. Any truncation or cleavage products that arise during in vitro RNA synthesis or labeling will be visible on the high-resolution acrylamide gels used. In addition, gel-purification completely removes free radioactive nucleotides, allowing more precise determination of the specific activity (counts per minute) of radiolabeled RNA.

3.2.1 Preparation of Lead Cleavage Reactions

1. RNP assembly reaction.
   (a) sR8 or sR6 box C/D RNA (or RNA of interest) (1×10^5 cpms, 2 pmol).

(b) 20 pmol L7Ae, 32 pmol Nop56/58, 32 pmol fibrillarin (or proteins of interest).

(c) 1 μL 10× Assembly Buffer.

(d) 0.5 μL tRNA (10 mg/mL).

(e) ddH_2O up to 8.5 μL.

2. Heat reaction to 70 °C for 8 min (or appropriate temperature for RNP assembly) (*see* **Note 1**) and then cool to room temperature.
3. Add 0.5 μL of 40 μM target RNA complementary to sR8 or sR6 (or RNA of interest) where applicable. Heat to 50 °C for 3 min and cool to room temperature (*see* **Note 2**).
4. Add 1 μL of 20 mM lead acetate (*see* **Note 3**) and incubate at 23 °C for 8–12 min.
5. Stop cleavage reaction by addition of 90 μL of 2 % $LiClO_4$ in acetone (*see* **Note 4**).
6. Pellet RNA by centrifugation at 10,000 × *g* for 5 min. Wash pellet with acetone, then pellet again with centrifugation. Aspirate acetone and air dry pellet in tube.
7. Prepare T1 RNase cleavage ladder (cuts after every accessible guanine nucleotide).

(a) sR8 or sR6 RNA (or RNA of interest) (1×10^5 cpms, 2 pmol).

(b) 1 μL 10× Assembly Buffer.

(c) 1 μL RNase T1 (0.5 U/μL).

(d) ddH_2O up to 10 μL.

8. Incubate T1 RNase ladder reaction for 4 min at 23 °C. Optimal reaction times may need to be determined empirically.
9. Stop reaction and pellet RNA using 2 % $LiClO_4$ in acetone as described above.
10. Prepare alkaline hydrolysis ladder (cuts after every nucleotide).

(a) sR8 or sR6 RNA (or RNA of interest) (1×10^5 cpms, 2 pmol).

(b) 1 μL 10× Alkaline Hydrolysis Buffer.

(c) ddH_2O up to 10 μL.

11. Incubate reaction at 95 °C for 8 min. Optimal reaction times may need to be determined empirically.
12. Stop reaction and pellet RNA using 2 % $LiClO_4$ in acetone as described above.
13. Store all washed and dried RNA pellets at –20 °C.

3.2.2 Resolution of Cleavage Products on a Denaturing Polyacrylamide Sequencing Gel

Casting the Denaturing Polyacrylamide Sequencing Gel

1. Wash glass plates, spacers, and comb with soapy water. Rinse with ddH_2O and wipe surface dry with 100 % EtOH (*see* **Note 5**).
2. Assemble plates with spacers and clamp together on the sides with large spring clips. Do not place a spacer at the bottom. Leave bottom open so that the gel solution can flow out if needed.
3. Lay assembled glass sandwich across two pipette tip boxes, with the box near the top of the gel assembly also having a standard microfuge tube rack on top (~2.5 cm tall) such that the entire assembly is at a ~15° incline. This will allow the gel solution to flow evenly from the top of the gel to the bottom when pouring.
4. In a 50 mL screw-top conical tube prepare 35 mL of gel solution: 14 % acrylamide (19:1), 7 M urea, 1× TBE, 4 % glycerol (*see* **Note 6**).
5. Add 35 μL TEMED, mix well, then add 210 μL of fresh 10 % APS. Mix quickly.
6. Pour polyacrylamide solution between plates starting at the top of the assembly. Tap on the glass plates while pouring to prevent bubble formation as the solution moves toward the bottom by capillary action.
7. Place gel plates level on gel boxes, remove top two clips on the assembly, insert comb slowly to avoid bubble formation, then reattach clips. Add clips to hold the comb and allow 30–60 min for polymerization.

Resolving Lead Cleavage Products on a Sequencing Gel

A sequencing gel can separate RNA species at nucleotide resolution, which is ideal for resolving RNA cleavage products. When combined with RNase T1 and alkaline hydrolysis ladders, it also allows for precise mapping of cleavage sites on the RNA sequence. During heavy metal treatment of RNA, cleavage sites are typically interpreted as flexible and non-constrained bases, indicating a lack of base pairing or protein binding. Sites protected from cleavage often indicate constrained bases, such as those involved in base pairing interactions.

1. Carefully remove comb and rinse sample wells with ddH_2O.
2. Mount gel assembly onto electrophoresis apparatus, fill top and bottom tanks with 1× TBE, then electrophorese gel at a constant power of 55 W for 45 min (*see* **Note 7**).
3. Dissolve RNA pellets from digestion reactions with 8 μL of Loading Buffer at 90 °C for 3 min.
4. Stop sequencing gel pre-electrophoresis, rinse out the wells with 1× TBE, then load samples into wells using a pipetman with flat loading tips.
5. Continue gel electrophoresis at the same setting (55 W) for approximately 2 h (*see* **Note 7**).

Drying the Sequencing Gel and Visualizing Radioactive RNA Cleavage Products

1. Turn off the power supply, remove gel assembly from electrophoresis apparatus and lay it flat on the lab bench.
2. Pry apart plates slowly from one corner of the assembly using a wedged object (Hoefer Wonder Wedge®). The gel should stick to the bottom or top glass plate (*see* **Note 8**).
3. Lay plate with gel attached flat on lab bench with the gel facing upwards.
4. Wet the edge of a thin-wedged piece of plastic (Hoefer Wonder Wedge® or gel spacer) with a few drops of 30 % acetic acid/20 % ethanol then loosen all four sides of the gel from the glass plate by running this plastic along the edge. If edges are not released, gel tearing may ensue.
5. Gently layer thin plastic wrap over the entire gel. Flip the gel over so that the glass plate is on top and the polyacrylamide gel is on the bottom against the bench with the plastic wrap underneath.
6. Lift one edge of the glass plate and slowly separate the gel from the glass with a Wonder Wedge®. Free an entire edge of the gel, then slowly pull the plate up while holding the plastic wrap and gel down close to the bench. The gel should stick to the plastic wrap (*see* **Note 9**).
7. Lay two sheets of 3MM Whatman filter paper on top of the gel. Carefully flip gel over and fold over excess plastic wrap or cut it off.
8. Dry the gel on large vacuum gel dryer apparatus at 80 °C for 2 h with gel facing up (paper on bottom, plastic wrap on top).
9. Discard the bottom sheet of filter paper in radioactive waste. Cut dried gel down to size to remove excess lanes that did not contain sample. Expose the gel in a large phosphorimager cassette overnight or directly on a large sheet of X-ray film for autoradiography for ~3 h. Exposure time to film may need to be determined empirically.

4 Notes

1. *M. jannaschii* is a thermophile and efficient binding of the core protein to box C/D RNA requires elevated temperatures. Most RNA-binding proteins bind optimally in a range from 4 to 37 °C. Frequently, a carrier protein, such as bovine serum albumin (BSA) is added in excess to keep protein levels at a relatively constant concentration, prevent protein precipitation, and provide clearer RNP resolution. BSA is not included in these reactions due to aggregation and precipitation that can occur under the extreme heat conditions employed for protein binding.

2. Annealing of the target RNA is performed at 50 °C because the RNP assembled in this protocol uses thermophilic proteins. Typically, target RNA binding would occur at temperatures at or below that of the RNP assembly temperature. For mesophilic proteins, where RNP assembly is usually performed at 4–37 °C, the target RNA may be added at or below the RNP assembly temperature. Optimal target RNA binding parameters will need to be determined for each RNP under investigation.
3. The lead acetate solution should be made fresh or stored at –20 °C in single-use aliquots. Lead is a dangerous heavy metal and precautions outlined in the MSDS should be carefully followed. Cleavage time may need to be determined empirically for the particular RNAs and RNPs being studied. When performing cleavage in multiple reactions, addition of lead acetate should be staggered, such as by 15 s intervals. Reactions should then be stopped in the same order with the same staggered time.
4. Lithium perchlorate is an ideal precipitation reagent for RNA cleavage and mapping reactions. It efficiently precipitates even single nucleotides in very dilute solutions, thus allowing near complete recovery of even the smallest cleavage products. However, be sure to wash pellet with acetone in the following step so that excess salt is not carried over in the RNA samples. Excess salt will distort the resolved RNA fragment pattern as a consequence of creating a salt front during electrophoresis.
5. Water should run off the glass plates in sheets if they are truly clean. Absolutely clean plates are essential when pouring the polyacrylamide gel. Do not use coatings like RainX® or Sigmacote® which are hard to remove and can interfere with capillary action during the gel pouring or gel removal for drying. This is primarily because sequencing gels are extremely thin. To remove such coatings, as well as to thoroughly clean glass plates in general, treat with a nitric acid solution overnight then rinse copiously with water. Note which side of the glass plates are facing each other and touching the gel. Always try to use the same sides of the glass plates that face and touch the gel. These sides of the glass plates tend to become quite clean over time due to urea contained in the gel and elevated electrophoresis temperatures.
6. Gel percentages should be chosen based upon the size of the RNA fragments to be resolved. For the box C/D RNAs in this protocol, 12–14 % polyacrylamide worked well. For smaller or larger RNAs, higher or lower percentages of polyacrylamide should be used, respectively. Glycerol is an important component of the sequencing gel and should not be omitted. During gel drying prior to visualization, glycerol

prevents the polyacrylamide gel from shrinking and cracking, which can make a gel unusable for visualizing the RNA fragment pattern as well as ultimate publication.

7. 55 W is a suitable power setting for both pre-electrophoresis and electrophoresis. The gel plates should be very warm or hot to the touch during electrophoresis. However, if the plates get too hot they will crack or shatter. Gel pre-electrophoresis is necessary to warm the gel, condition it by removing excess ammonium persulfate left over from polymerization, and ultimately minimize sample "smiling." The heat and urea in the gel help to keep the RNA denatured. As a general rule, electrophorese the digested RNA until the dye front has migrated approximately one-third of the way through the gel. It is important to be able to visualize the uncut RNA band at the top of the gel later and also see most or all of the RNA cleavage products including the smallest fragments. Tables of dye migration for bromophenol blue and xylene cyanol dyes in different concentrations of polyacrylamide are available online (http://www.invitrogen.com/site/us/en/home/References/Ambion-Tech-Support/rna-electrophoresis-markers/general-articles/gel-electrophoresis-tables.html) to help determine electrophoresis times.
8. Sequencing gels are extremely thin and can tear easily. To avoid sticking to both plates, watch the gel as you slowly pry apart the glass plates. If the gel appears to be sticking to both plates, press the plates back together, press down on the plates, and then try again to pry apart the plates using another corner of the gel. Alternatively, judicious amounts of 30 % acetic acid, 20 % ethanol solution applied from a squirt bottle in combination with the Wonder Wedge® should aid in getting the gel to stick to one plate or the other. This procedure may also be critical if the gel becomes folded upon itself when the glass plates are removed. Water should be avoided as it will cause the bands to diffuse out of the gel, particularly the smallest fragments. Thus, even if the gel is successfully unfolded and placed on a single plate, the gel image will likely be of poor quality. In contrast, acetic acid/ethanol will fix the RNA into the gel and prevent diffusion while providing lubrication to manipulate the gel without tearing. The use of acetic acid/ethanol solution and the Wonder Wedge® will also help prevent tearing and folding of the gel during transfer from the glass plate to the plastic sheeting.
9. Refer to **Note 8** above for help in transferring the polyacrylamide gel from the glass plate to plastic wrap for drying. To avoid tears along the edges, use a Wonder Wedge® or thin piece of plastic to release the gel from the glass. As an alternative to drying the gel, X-ray film or a phosphorimager cassette may be placed directly on the gel once the plastic wrap has

been applied. The RNA image can be revealed by exposure overnight in a dark cold room (phosphorimager cassette and gel wrapped in black plastic bag) or exposed 3–5 h in a –80 °C freezer (autoradiography cassette with X-ray film, wrapped in black plastic bag or aluminum foil).

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