Electronic Supplementary Material 2

Analysis of the RNA Backbone: Structural Analysis of Riboswitches by In-Line Probing and Selective 2′-Hydroxyl Acylation and Primer Extension (SHAPE)

Catherine A. Wakeman and Wade C. Winkler

Abstract

RNA sequences fold upon themselves to form complex structures. Functional analysis of most biological RNAs requires knowledge of secondary structure arrangements and tertiary base interactions. Therefore, rapid and comprehensive methods for assessing RNA structure are highly desirable. Computational tools are oftentimes employed for prediction of secondary structure. However, a greater degree of accuracy is achieved when these methods are combined alongside structure probing experimentation. Multiple probing techniques have been developed to assist identification of base-paired regions. However, most of these techniques investigate only a subset of RNA nucleotides at a time. A combination of structure probing approaches is thus required for analysis of all nucleotides within a given RNA molecule. Therefore, methods that investigate local structure for all positions in a sequence-independent manner can be particularly useful in characterizing secondary structure and RNA conformational changes. This chapter outlines protocols for two techniques, in-line probing and Selective 2′-Hydroxyl Acylation Analyzed by Primer Extension (SHAPE), which largely accomplish this goal.

Key words: Riboswitch, RNA folding, RNA secondary structure, RNA structure analysis, in-line probing, SHAPE.

1. Introduction

Riboswitches are RNA molecules that function as direct sensors for intracellular metabolites and metal ions (1–5). They are typically located within the 5′-untranslated region (5′-UTR) of the mRNA that hosts them and are responsible for regulation of the downstream genes in response to fluctuations of their metabolite

---

or metal ion ligand(s). Specifically, association of the target metabolite (or metal ion) to the ligand-binding portion (aptamer) stabilizes an RNA conformation that either induces or represses expression of downstream gene(s) (2–5). Functional analyses of riboswitch RNAs have greatly benefited from identification of secondary structure and tertiary base interactions. This has been largely accomplished through a combination of bioinformatics-based approaches and structural probing techniques. These methods have directly assisted in the analyses of nucleotides that are involved in ligand recognition, estimation of apparent K_D values, and in characterizing ligand-induced structural rearrangements (6, 7). Moreover, these predictions of secondary structure have largely been validated by the three-dimensional analyses of riboswitch aptamer domains by X-ray crystallography and NMR (2–5).

In total, there are a variety of routinely used RNA structure probing techniques, each exhibiting particular strengths and weaknesses. One method of characterizing secondary structure is to partially digest the RNA substrate with RNase enzymes that exhibit specific substrate preferences (8). For example, nucleotides that are single-stranded can be inferred by partial digestion of the target RNA by RNase S1, which prefers such sites. Similarly, unpaired Gs can be identified by partial digestion with RNase T1. Conversely, base-paired and stacked nucleotides can be indicated via RNase V1 cleavages. Combined, these individual enzymatic reactions can assist in the identification of helices or unpaired nucleotides. Chemical agents may also be employed for structural probing purposes, such as cleavage of single-stranded regions via lead probing or methylation of unpaired guanine N1 and N2 positions by kethoxal (9, 10). Finally, nucleotide analog interference mapping (NAIM) is a powerful method of rapidly investigating the effect(s) of substituting specific nucleotide functional groups (11). However, methods that investigate local structure in a sequence-independent manner are particularly useful in predicting RNA secondary structure and for analyzing conformational changes. Two such methods, briefly described herein, are referred to as in-line probing and Selective 2′-Hydroxyl Acylation Analyzed by Primer Extension (SHAPE) (12–14). In-line probing provides a measurement of the relative flexibility of specific RNA linkages due to the inherent differences in their rates of spontaneous cleavage (12, 15). Linkages that exhibit greater "flexibility" (i.e., unpaired status) exhibit increased rates of spontaneous cleavage. Therefore, comparative analysis of the cleavage rates for all linkages of a given RNA molecule can be used to identify unpaired and base-paired regions. SHAPE also probes the relative flexibility of RNA linkages. Specifically, the nucleophilic character of the 2′ ribose hydroxyl is influenced by its proximity to the adjacent 3′-phosphodiester anion,

which is in turn governed by the local structural context. Therefore, reactivity of the 2′ ribose hydroxyl to an electrophile, e.g., *N*-methylisatoic anhydride (13), is sensitive to local structure. Similar to in-line probing, SHAPE can assist in monitoring the local structure for every position in a given RNA molecule in a sequence-independent manner.

2. Materials

2.1. Preparation of RNA by In Vitro Transcription

1. T7 RNA polymerase; store at –20°C (*see* **Note 1**).
2. DNA template generated by PCR-amplification using a forward primer that incorporates the T7 promoter sequence (TAATACGACTCACTATA*GGG*).
3. 10× transcription buffer: 300 mM Tris–HCl, pH 8.0, 100 mM DTT, 1% Triton X-100, 1 mM spermidine, 400 mM $MgCl_2$.
4. 25 mM NTP mix: 25 mM rATP, rCTP, rGTP, and rUTP (Roche) – store at –20°.
5. Optional: yeast inorganic pyrophosphatase (Sigma) (*see* **Note 2**).
6. 2× urea loading buffer: 10 M urea, 1.5 mM EDTA, pH 8.0, 10 mM Tris–HCl, pH 8.0, 0.05% bromophenol blue, 0.05% xylene cyanol.
7. Crush-soak solution: 200 mM NaCl, 10 mM Tris–HCl, pH 7.5, and 1 mM EDTA. Filter-sterilize the solution.
8. This section assumes the use of fluor-coated thin layer chromatography (TLC) plates and a hand-held device for short-wave UV light (254 nm).

2.2. Radiolabeling of Nucleic Acids at the 5′-Terminus

1. ~10–50 picomoles synthetic RNA per ~13.3 picomoles [γ-^{32}P]ATP.
2. Calf intestinal alkaline phosphatase (CIP) (New England Biolabs).
3. T4-polynucleotide kinase (PNK) (New England Biolabs).
4. [γ-^{32}P]ATP (Adenosine 5′-triphosphate), 6,000 Ci/mmole on reference date.
5. 5× kinase buffer: 25 mM $MgCl_2$, 125 mM CHES, pH 9.0, 15 mM DTT (*see* **Note 3**).
6. Phenol:Chloroform:Isoamyl alcohol 25:24:1 (v/v/v).
7. Chloroform.
8. Optional: glycogen (20 mg/mL) (Roche, *see* **Note 4**).

9. 3 M sodium acetate, pH 5.2.

10. Autoradiography film.

2.3. In-Line Probing

1. 5′-terminus radiolabeled RNA; store at –20°C for up to 2 weeks (*see* **Note 5**).

2. 2× in-line/SHAPE buffer: 100 mM Tris–HCl, pH 8.3, 200 mM KCl, 40 mM $MgCl_2$ (*see* **Notes 6 and 7**).

3. RNase T1 (Sigma) diluted to 4 units/μL in H_2O; store at 4°C.

4. 10× T1 buffer: 0.25 M sodium citrate, pH 5.0.

5. 10× OH buffer: 0.5 M Na_2CO_3, pH 9.0, 10 mM EDTA, pH 8.0.

2.4. SHAPE Probing

1. Non-radioactively labeled RNA; store at –20°C.

2. 5′-radiolabeled DNA oligonucleotide (~15–30 nucleotides) that is complementary to the 3′-end of the target RNA; store at –20°C for up to 2 weeks.

3. 65 mM *N*-methylisatoic anhydride (NMIA) (Invitrogen) solution dissolved in dimethyl sulfoxide (DMSO) (Sigma); prepare fresh (*see* **Note 8**).

4. Transcriptor reverse transcriptase (Roche); store at –20°C (*see* **Note 9**).

5. dNTPs solution: 10 mM dATP, dCTP, dGTP, and dTTP (Roche); store at –20°C.

6. 4 M NaOH.

7. 10× TBE: 108 g Tris, 55 g boric acid, and 3.725 g EDTA in 1 L H_2O.

8. Acid stop buffer: Make acid stop dye (85% formamide (v/v), 0.5× TBE, 50 mM EDTA, pH 8.0, 0.05% bromophenol blue, and 0.05% xylene cyanol). Mix the acid stop dye 25:4 (v/v) with 1 M unbuffered Tris base; store at –20°C.

9. Thermosequenase cycle sequencing kit (USB) or other method for generation of DNA sequencing ladders via dideoxynucleotide incorporation.

2.5. Polyacrylamide Gel Electrophoresis (PAGE)

1. 37% Acrylamide/bis-acrylamide 29:1 (w/w): dissolve 89.5125 g acrylamide and 3.0875 g bis-acrylamide in water, and bring up to a final volume of 250 mL. Filter through Whatman paper. Store in amber bottles at 4°C (*see* **Note 10**).

2. Urea.

3. Running buffer: 1× TBE (10× TBE diluted 1:10 in H_2O).

4. Ammonium persulfate (APS): Make a 10% solution (w/v) with H_2O; store at 4°C for up to a month.

5. N,N,N′,N′-tetramethylethylenediamine (TEMED); store at 4°C.

6. This section assumes the use of glass plates that are 32.5 cm × 41 cm with 0.75-mm spacers and 24-well combs for in-line probing and SHAPE gels. We typically use 28 cm × 16.5 cm glass plates with 0.75-mm spacers and 4- to 8-well combs for RNA preparative techniques (*see* **Note 11**).

7. Vacuum pump and gel dryer.

8. Whatman paper, 3 mm chromatography paper.

9. 35 cm × 43 cm phosphor screens (Amersham).

10. Phosphor imaging instrumentation, e.g., Typhoon 9200 Variable Mode Imager, and software (Molecular Dynamics).

3. Methods

RNA phosphodiester linkages are spontaneously cleaved as the result of an S_N2 attack by the ribose 2′ oxygen on the adjacent phosphorus (12). This attack results in a 5′ cleavage fragment with a 2′,3′-cyclic phosphate and a 3′ cleavage fragment with a 5′ hydroxyl terminus. The rate at which this reaction occurs is dependent upon the degree of "in-line" positioning of the 2′ oxygen, phosphorus, and 5′ leaving group oxygen atoms for a given RNA internucleotide linkage (16–19). Maximal rates are achieved when the 2′ oxygen, the phosphorus center, and the 5′ oxygen leaving group form a perfect 180°. This arrangement is required for a productive nucleophilic attack by the 2′ oxygen. Linkages for nucleotides that participate in stable base-pairs exhibit lower rates of spontaneous cleavage than for nucleotides that are located in relatively unstructured regions (12, 18). Thus, spontaneous cleavage of phosphodiester linkages is highly dependent upon the local structural context. To exploit this for the prediction of RNA secondary structure, a radiolabeled phosphorus group is placed at either the 5′ or 3′ terminus of the target RNA. These radiolabeled RNAs are then incubated under desired reaction conditions for a period of time (generally ~40 h) that is long enough for spontaneous self-cleavage to occur for a small subset of the total population of RNA molecules (12). The resulting RNA fragments are then resolved by denaturing gel electrophoresis alongside appropriate sequencing ladders and analyzed by phosphor imaging instrumentation. Bands of greater intensity correlate with "flexible" RNA linkages, which are more capable of randomly adopting a near perfect in-line conformation. Conversely, portions of the gel that appear to lack bands

correlate with positions that are likely to be involved in base-pairing interactions. The reaction components for in-line probing can be significantly varied with only modest effects upon the assay results. Temperature of the reactions can also be modestly varied, although the overall incubation times must be adjusted accordingly (12, 20). Changes in magnesium concentration can modestly affect the band intensity for all reactive positions. However, magnesium can be removed from the reactions with a modest reduction in spontaneous cleavage (12). In prior studies, our laboratory has titrated magnesium into in-line probing reactions while maintaining high monovalent cation concentrations in order to characterize a magnesium-sensing riboswitch RNA (21). Under these conditions the influence of magnesium on the rates of RNA spontaneous cleavage is greatly minimized. In the past, experimentation very similar to in-line probing as described herein have been interpreted as demonstrating that addition of magnesium to RNA leads to high rates of spontaneous cleavage for backbone linkages adjacent to specific magnesium binding sites. We recommend using caution when arriving at these conclusions. Sites of spontaneous cleavage observed by in-line probing reactions (in the presence of magnesium) do not necessarily correlate well with highly occupied magnesium-binding pockets that have been identified by structure-determining methods such as X-ray crystallography. In contrast, the in-line character of RNA linkages in three-dimensional structures has been shown to agree well with the relative rates of RNA cleavages observed during in-line probing (12). Additionally, as mentioned above, magnesium can be removed from in-line probing reactions under certain conditions with only a minor influence on overall spontaneous cleavage rates (12). More detailed discussion on any potential correlation between metal-binding sites and cleavages at specific linkages, a subject that is not without debate, is beyond the scope of this protocol outline.

Similar to in-line probing, SHAPE is an unbiased method for distinguishing base-paired and single-stranded regions of RNA in a sequence-independent manner. Originally developed by Kevin Weeks' laboratory, several versions of the SHAPE probing protocol have been published elsewhere (13, 14, 22). SHAPE probing is built upon the observation that the reactivity (i.e., nucleophilic character) of the ribose 2′-hydroxyl is influenced by local nucleotide flexibility. For nucleotides constrained by base pairing interactions, close proximity of the 3′-phosphodiester anion reduces reactivity of the 2′-hydroxyl. This corresponds with lower rates of reactivity to an appropriate electrophile, such as *N*-methylisatoic anhydride (NMIA), for formation of a 2′-*O*-adduct. However, flexible linkages allow for conformations that exhibit increased reactivity to NMIA. Sites of 2′-*O*-adduct formation can then be detected via disruption of cDNA synthesis by reverse

transcriptase. Importantly, NMIA reactions are self-quenching by virtue of the fact that NMIA undergoes a parallel self-inactivating reaction (13). For these reasons, SHAPE is an experimentally easy, robust and rapid method for prediction of paired and single-stranded regions within a target RNA.

Both in-line probing and SHAPE analysis have been employed to investigate riboswitch RNAs in the presence and absence of their ligands. These analyses have revealed the overall secondary structure patterns for riboswitch RNAs and highlighted ligand-induced structural changes. When repeated at a range of ligand concentrations, both methods can be used to determine the apparent dissociation constants (K_D) for RNA-ligand interactions. Not restricted to metabolite-binding RNAs, these methods are generally applicable for analysis of RNA structure and the study of RNA-ligand interactions.

3.1. Preparation of RNA by In Vitro Transcription

1. For a yield of >200 pmols of RNA, combine ~20–50 pmol DNA template, 2.5 µL 10× transcription buffer, 2.5–5 mM each NTP, ~50 µg/mL T7 RNA polymerase, and 0.0025–0.01 U inorganic pyrophosphatase (optional) in a final volume of 25 µL. These reactions can be scaled appropriately for recovery of the desired quantity of synthetic RNA. The reaction should be incubated for 2–3 h at 37°C (*see* **Note 12**) and terminated by the addition of an equal volume of 2× urea loading buffer (*see* **Note 13**). The transcription reaction can be stored at –20°C at this point.

2. The RNA should be resolved by, and excised from, a denaturing polyacrylamide gel (as detailed in **Section 3.5**) (*see* **Note 14**). The polyacrylamide percentage (6–15%) should be chosen as deemed appropriate for the length of the RNA substrate.

3. Once the bromophenol blue dye marker has run ~2/3 the length of the plates, electrophoresis can be terminated. At this point, the glass plates containing the gel should be removed from the gel rig. To remove the gel from the glass plates, slide out the spacers, lay the glass plates flat on the bench top, and carefully pry them apart. The gel will typically preferentially stick to one plate. Flip the sandwich over so that the gel-associated plate is on the bottom and continue with the separation. Once one plate is removed, place plastic wrap over the exposed side of the gel. Flip the gel and gently separate the plastic wrap-associated gel from the remaining plate. Cover the newly exposed side of the gel with plastic wrap.

4. The synthetic RNA can then be visualized by UV shadowing. Place the plastic wrap-enclosed gel sandwich over a TLC plate and briefly expose to shortwave UV light.

UV-absorbing material such as RNA polymers and free nucleotides will appear as dark shadows. Outline the topmost shadow with a fine-point marker as it should correspond to the target RNA. The lowest migrating band will likely represent the free nucleotides.

5. Excise the circled region with a razor blade, remove the outer layer of plastic wrap, and cut the gel slice into ~1 mm squares.

6. Place the gel bits into a 1.5-mL microcentrifuge tube and add approximately two volumes of crush-soak solution (typically ~400–600 µL). Incubate on a tube rotator at room temperature for 2 h or at 4°C overnight. Remove and save supernatant.

7. Ethanol precipitate the RNA by adding ~2.5 volumes cold 100% ethanol and incubating at –20°C for 30 min. Pellet the RNA by centrifuging at $20,000 \times g$ for 15 min. Wash pellet with 200 µL 70% ethanol and centrifugation at $20,000 \times g$ for 5 min. Carefully remove supernatant and air dry the pellet air for 1–5 min or by speedvac.

8. Resuspend the RNA in ~20–100 µL H_2O and quantify RNA yield via calculation of extinction coefficient values and measurement of A_{260}. Store at –20°C until use.

3.2. Radioactive-Labeling of Nucleic Acids at the 5′-Terminus

1. Prior to radioactive labeling, the RNA must first be dephosphorylated at the 5′-terminus. The 5′-terminus of DNA oligonucleotides produced by solid-phase synthesis typically does not contain phosphate groups. Therefore, skip to **step 4** for radiolabeling of commercially obtained DNA oligonucleotides. Combine ~10–40 pmols RNA and the commercial buffer for CIP in a 10 µL final volume and incubate at 50°C for 15 min.

2. Remove CIP by phenol:chloroform:isoamyl alcohol extraction. First, increase the volume of the reaction to 200 µL with H_2O. Add 200 µL phenol:chloroform:isoamyl alcohol and shake or vortex for ~5 s to mix completely. Centrifuge at $20,000 \times g$ for 5 min to separate into two phases. Remove the top, aqueous phase, which contains the RNA, and discard the bottom phase containing the protein. Repeat this procedure with 200 µL chloroform to remove traces of phenol.

3. Concentrate the RNA via ethanol precipitation. Add 1/10 volume (20 µL) 3 M sodium acetate and 1 µL glycogen to the RNA and mix. (*see* **Note 4**) Add 2.5× volume (500 µL) 100% ethanol, mix by inversion, and incubate at –20°C for 30 min. Pellet the RNA by centrifugation at $20,000 \times g$ for 15 min. The pellet should then be washed by the addition

of 200 µL 70% ethanol and centrifuged at 20,000 × *g* for 5 min. Discard supernatant, air dry or speedvac the pellet for 1–5 min, and resuspend the RNA in 10 µL H_2O.

4. Once the RNA has been dephosphorylated, a radiolabeled phosphate group can be transferred to the 5′-terminus using T4 PNK. Per 20 µL kinase reaction employ 5 µL of CIP-treated RNA, 4 µL 5× kinase buffer, 4–12 µL [γ-^{32}P]ATP, and 2 µL T4 polynucleotide kinase (PNK) at 10 U/µL. Increase the final volume to 20 µL with H_2O. Incubate the reaction for 35 min at 37°C. For 5′-radiolabeling of DNA oligonucleotides set up identical reactions except with 10–50 pmol DNA.

5. The resulting 5′-radiolabeled nucleic acids can then be resolved by denaturing polyacrylamide gel electrophoresis. Use 6% PAGE for nucleic acids greater than 75 nucleotides and 10% for nucleic acids less than 75 nucleotides (as detailed in **Section 3.5** and **step 3** of **Section 3.1**). Prior to discarding, buffers in the upper and lower reservoirs of the gel rig should be checked for radioactivity.

6. Remove the polyacrylamide gel from the glass plates and place between layers of plastic wrap. Radioactively labeled bands can then be identified via brief exposure to autoradiography film. The gel should be secured inside the film cassette so that it cannot move and can be reproducibly positioned against the autoradiography film. A sheet of autoradiography film should be exposed to the gel for ~1 min and developed. Outline the region of the gel sandwich that contains the radiolabeled RNA as identified by dark band(s) on the autoradiography film.

7. Excise the RNA from the gel using the procedure detailed in **Section 3.1 steps 5, 7, and 8**.

3.3. In-Line Probing

1. In a final volume of 10 µL, combine 2× in-line/SHAPE buffer, ~75–200 kcpm 5′-radiolabeled RNA, and the desired amount(s) of any other substance to be included in the assays (e.g., RNA-binding protein or metabolite). Incubate at 22–25°C for ~40 h (*see* **Note 15**). This will allow for spontaneous cleavage of a subpopulation of the RNAs via single-hit kinetics (12). Terminate the reaction by addition of 10 µL of 2× urea loading buffer and immediate storage at –20°C. Resolve the RNA fragments by denaturing polyacrylamide gel electrophoresis alongside size marker ladders. There are many different size markers that could be used. Our laboratory typically includes lanes containing RNAs that have been partially digested with RNase T1 to visualize guanosine residues and a lane for RNAs that were briefly

exposed to increased pH and high temperature for visualization of cleavage at all nucleotide positions. We refer to these size marker control reactions herein as "T1" and "-OH" ladders, respectively.

2. Prepare the T1 ladder by mixing 1 μL 5′-radiolabeled RNA (~100 kcpm – similar in quantity to the experimental lanes) with 1 μL RNase T1 (4 U/μL) and 1 μL 10× T1 buffer and increase the volume to 10 μL with 2× urea loading buffer. Incubate at 50°C for 20 min. The most appropriate reaction times will need to be optimized per target RNA. Add 3 μL 2× urea loading buffer and 7 μL H₂O and store at –20°C prior to resolution by denaturing PAGE.

3. Prepare the -OH ladder by mixing ~100 kcpm 5′-radiolabeled RNA with 1 μL of 10× OH buffer and increase the volume to 10 μL with H₂O. Incubate at 95°C for 3–8 min to induce scission after every base (the appropriate time interval required for these reactions will need to be optimized per target RNA). Stop reaction with 10 μL 2× urea loading buffer and immediately store at –20°C.

4. Resolve the in-line probing reactions and size marker ladders by denaturing 6–20% polyacrylamide gel electrophoresis (the most appropriate polyacrylamide percentage should depend on the size of the target RNAs) next to ~100 kcpm non-reacted (NR) radiolabeled RNA in 10 μL 2× urea loading buffer, following the protocol described in **Section 3.5**.

5. Results (**Fig. 2.1**) from these assays should resemble representative reactions shown in **Fig. 2.1a, c, e**. When analyzing the data, consider bands to be regions of the RNA that are flexible/unstructured such as loops and bulges. Clearings on the gel can be considered regions of RNA, such as helices, that are structurally constrained by base interactions (*see* **Note 16**). The location of these regions can be mapped onto the RNA sequence using the T1 and OH ladders as shown in **Fig. 2.1b, d**. The T1 ladder will reveal the location for guanosine residues within the RNA while the OH ladder displays bands for every nucleotide. The non-reacted RNA demonstrates the quality of the RNA prior to treatment and should display minimal to no banding. For more detail on data analysis, see **Section 3.6**.

3.4. SHAPE Probing

1. In a final volume of 18 μL, combine 10 μL 2× in-line/SHAPE buffer, 2 pmol RNA, and the desired concentration of potential ligand to be tested (*see* **Note 17**). Incubate for 1 h at room temperature to allow the RNA to equilibrate with its potential ligand and for RNA folding (*see* **Note 18**).
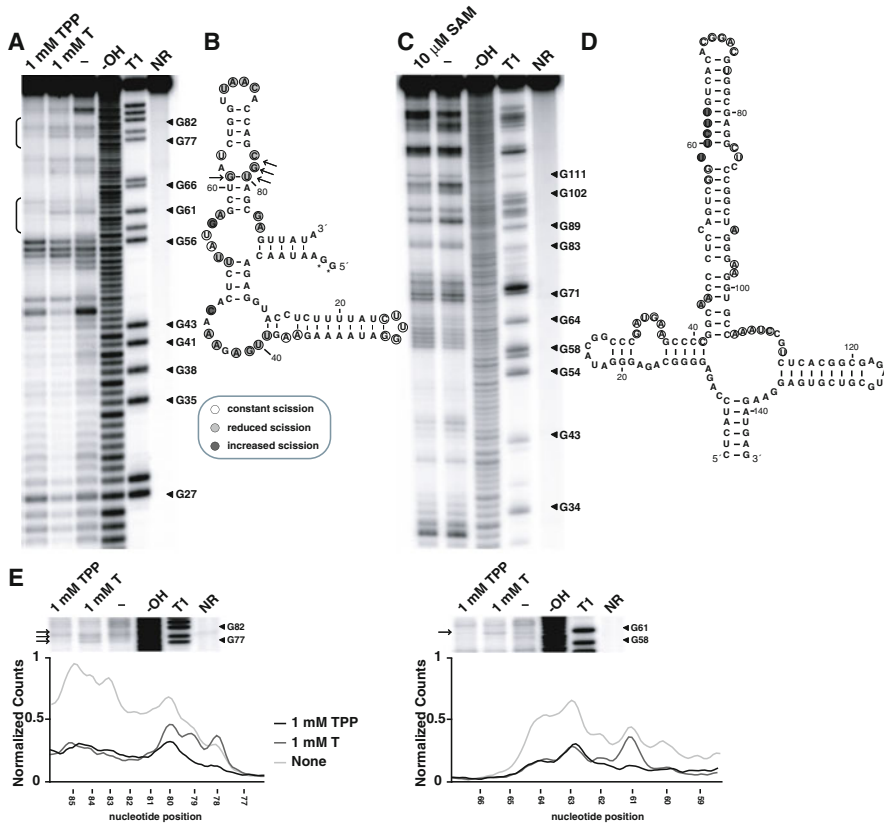
Fig. 2.1. Representative in-line probing of metabolite-binding RNA elements. (**a**) In-line probing of the thiamine pyrophosphate (TPP)-binding riboswitch aptamer of the *Mycoplasma gallisepticum hatABC* leader region. These reactions were performed in the presence or absence of thiamine (T) or TPP, the preferred ligand, and were resolved by denaturing 10% PAGE. Binding of T induces conformational changes as evidenced by changes in intensity for multiple bands within the individual lanes. The gray curved lines denote the regions of this RNA that were analyzed by line traces in panel E of this figure. T1 and -OH lanes represent size marker ladders resulted from partial digestion of the RNA molecules by RNase T1, which cleaves after G residues, and alkali, which cleaves after every nucleotide, respectively. The NR lane includes non-reacted RNA to demonstrate the quality of the RNA prior to in-line probing. (**b**) Probing changes induced by addition of 1 mM TPP have been mapped onto the RNA secondary structure diagram. Open circles indicate regions of the RNA displaying a constant level of scission under all reaction conditions. Light gray and dark gray circles indicate regions of the RNA displaying decreased or increased levels of scission upon addition of TPP, respectively. RNA nucleotides that are likely to be involved in recognition of the diphosphate portion of TPP are denoted by arrows. (**c**) Representative in-line probing of a *Streptomyces coelicolor S*-adenosylmethionine (SAM)-binding riboswitch in the presence or absence of SAM. (**d**) Probing changes induced by SAM are summarized in relation to the secondary structure diagram. (**e**) Representative data showing detection of ligand recognition determinants using in-line probing. Line traces are shown for regions of the *Mycoplasma* TPP riboswitch highlighted in panel A. The light gray line results from the no-ligand control lane while the dark gray and black lines result from the lanes containing 1 mM T and 1 mM TPP, respectively. While both T and TPP induce a similar overall conformational change, in-line probing patterns revealed several bands that are reduced in intensity for reactions that included TPP but not T (27). These differences are highlighted by normalized line graphs shown beneath representative gel slices. Because T and TPP differ only by a diphosphate, these data suggest that the TPP-specific positions are likely to be required for recognition of ligand phosphates. Indeed, three-dimensional structures of TPP riboswitches confirmed these biochemical data (2, 3, 7).

2. At this point, the mixture should be split into two separate 9 μL reactions. Add 1 μL NMIA solution to one reaction aliquot and 1 μL DMSO to the remaining aliquot. The reaction containing DMSO alone serves as a control. Incubate these reactions at 37°C for 45 min (*see* **Note 19**).

3. Increase the volume to 200 μL with H_2O and terminate the reactions by ethanol precipitation of the RNA as described in **step 3** of **Section 3.2**.

4. The pellets should be resuspended in 11 μL H_2O that also contains ~150–200 kcpm of the 5′-radiolabeled DNA oligonucleotide (reverse complement to the 3′-end of the substrate RNA). Incubate the reactions at 65°C for 5 min followed by 20 min at 37°C.

5. Add 2 μL 10 mM dNTPs, 2 μL H_2O, and 4 μL commercial 5× Transcriptor buffer and incubate at 52°C for at least 1 min but no more than 5 min.

6. Add 1 μL Transcriptor reverse transcriptase to initiate cDNA synthesis. Each reaction should be incubated at 52°C for exactly 5 min. For these reactions, oligonucleotide primers are typically 18–25 nucleotides in length and contain a G or C at the 3′-terminus.

7. Termination the cDNA synthesis reactions through addition of 1 μL 4 M NaOH and incubation at 95°C for 5 min. This should completely hydrolyze the substrate RNA.

8. Neutralize this reaction by adding 29 μL acid stop buffer and store at –20°C for up to 2 weeks prior to resolution by denaturing PAGE.

9. Prepare DNA sequencing ladders using standard manufacturers instructions and ~50–100 kcpm of the 5′-radiolabeled DNA oligonucleotide that was employed for cDNA synthesis after NMIA modification.

10. Incubate DNA sequencing ladders and reaction samples to 95°C for 5 min prior to resolution by denaturing 10% PAGE.

11. The results (**Fig. 2.2**) from these assays should resemble representative reactions shown in **Fig. 2.2a, c**. When analyzing the data, consider bands to be regions of the RNA that are flexible/unstructured such as loops and bulges. Clearings on the gel can be considered regions of RNA such as helices that are structured. The location of these regions can be mapped onto the RNA sequence using the DNA sequencing ladders (*see* **Fig. 2.2b**). Reactions that included NMIA should be directly compared to control reactions lacking NMIA. The first ~20 nucleotides, proximal to the 5′-terminus, will be difficult to analyze due
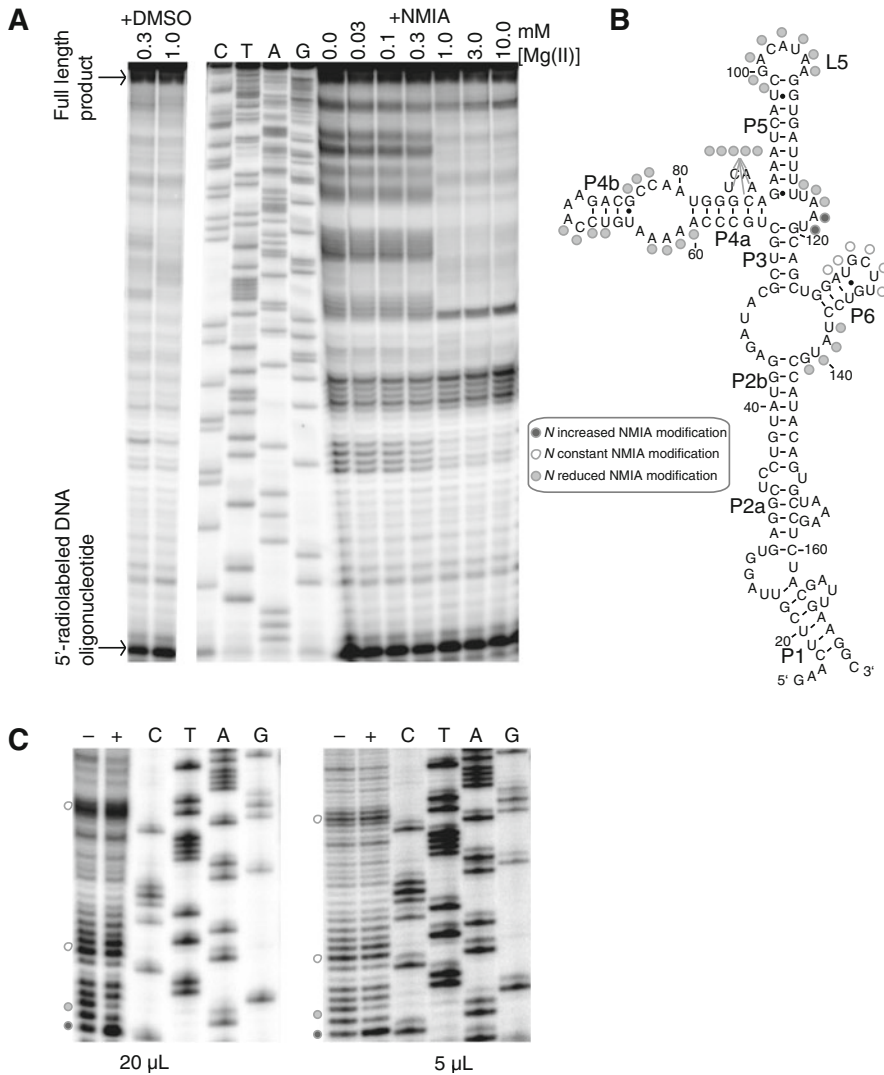
Fig. 2.2. Representative SHAPE probing for a riboswitch RNA. (**a**) SHAPE probing of a *B. subtilis* M-box RNA (magnesium-sensing riboswitch) at a range of magnesium concentrations. Bands of higher intensity correspond to RNA linkages that exhibit greater flexibility than for linkages constrained by base pairing. The +DMSO reactions did not include any NMIA and therefore show spontaneous RNA breakdown products and structure-induced reverse transcriptase pausing. These control reactions were performed at magnesium concentrations both above and below the presumed EC_{50} for the structural rearrangement. Sequencing ladders are resolved adjacent to the SHAPE reactions. (**b**) Results of SHAPE analysis are mapped onto the *B. subtilis* M-box RNA aptamer sequence. White circles indicate regions that exhibit constantly high levels of NMIA modification in all magnesium concentrations. Light gray and dark gray circles indicate regions that exhibit decreased (become more structured) or increased NMIA modification upon magnesium-binding, respectively. (**c**) SHAPE probing of a thiamine pyrophosphate (TPP)-binding aptamer in the presence or absence of 10 μM TPP. Representative gels were loaded with 5 or 20 μL aliquots to show reduced individual band resolution with large loading volume. TPP-induced NMIA modifications are coded similar to panel B.

to significant pausing exhibited by reverse transcriptase enzyme during initiation. For more detail on data analysis, *see* **Section 3.6**.

3.5. Polyacrylamide Gel Electrophoresis

1. Prepare 10% gel solution. In a 500 mL final volume, combine 240 g urea, 50 mL 10× TBE, and 135 mL 37% acrylamide/bis-acrylamide solution. Once the powder is dissolved, filter the solutions through Whatman paper. This solution can be stored in amber bottles at room temperature for up to a month. Adjust the volume of acrylamide/bis-acrylamide solution to achieve the desired polyacrylamide percentage.

2. To initiate polymerization of 100 mL of PAGE solution, gently add and mix 0.8 mL 10% APS and 0.04 mL TEMED, pour immediately, and slide in the comb. Allow the gel to polymerize for >30 min.

3. Gently remove the comb and rinse out the wells.

4. Assemble the gel electrophoresis rig and fill the upper and lower reservoirs with running buffer.

5. Pre-run the gel for 15 min. For glass plates that are 32.5 cm × 41 cm with 0.75 mm spacers we typically conduct electrophoresis at constant 60 watts. For glass plates that are 28 cm × 16.5 cm with 0.75-mm spacers we typically electrophorese samples at constant 40 watts.

6. Prior to loading the samples, rinse the wells thoroughly with running buffer.

7. For probing reactions, continue electrophoresis until the bromophenol blue indicator dye is ~1 inch from the bottom of the plate. For gels employed for preparative purposes we typically continue electrophoresis until the bromophenol blue dye has run ~2/3 the length of the plate, in order to retain free nucleotides within the gel.

8. For gels that are to be dried, remove one of the glass plates, allowing the gel to remain attached to the second plate. Press a sheet of Whatman paper on top of the exposed gel. The gel will adhere to the Whatman paper and can then be easily peeled away from the remaining glass plate. Cover the exposed side of the gel with plastic wrap and place in a gel dryer under vacuum pressure at 80°C for 2–3 h.

9. Expose the dried gel to a phosphor screen, which can then be analyzed via standard phosphor imaging instrumentation.

3.6. Analysis

1. This section assumes use of software resembling ImageQuant (Molecular Dynamics).

2. Each lane of the gel will exhibit a distinctive banding pattern. For in-line probing, the bands correspond to RNA

transcripts where the 3′-terminus has been generated by spontaneous cleavage of a flexible RNA linkage. Regions of the gel lacking bands are thereby indicative of RNA linkages that exhibit poor rates of spontaneous cleavage (e.g., base-paired positions). For SHAPE, the bands correspond to cDNAs containing a 3′-terminus at the site of NMIA modification. Since NMIA reactivity is dependent upon RNA linkage flexibility, the intensity of individual bands correlates with RNA linkage flexibility. Changes to the banding pattern between lanes are therefore suggestive of RNA conformational changes. Bands may darken, lighten, or disappear below detection. Dramatic banding changes can be easily observed by eye (*see* **Figs. 2.1 and 2.2**). This information can easily be structurally related to the RNA primary sequence through the aid of adjacent control reactions. For example, every SHAPE reaction should be resolved alongside DNA sequencing reactions, performed using the oligonucleotide primer also employed for cDNA synthesis of the NMIA-modified RNAs. For in-line probing, we find it convenient to include control reactions for cleavages at every position (alkaline-induced hydrolysis of the RNA backbone; "-OH") and cleavages at G residues (partial digestion by RNase T1; "T1"). Subtle changes in the banding pattern can be qualitatively analyzed by comparing the relative intensity profile of each lane. Specifically, a line trace can be drawn over the desired region of the gel and graphed, thereby generating a lane profile. Peaks and valleys correspond to bands and cleared regions, respectively. These data can be exported to spreadsheet analyses software such as Excel or SigmaPlot and carefully plotted and analyzed. Similar line traces copied onto control lanes, such as the T1 and –OH reactions, can be used to correlate line trace data to the overall RNA sequence. The line profile data can be normalized by converting the region of the each lane with the highest counts to 1 and the region with the lowest counts to 0.

3. Alternatively, a box can be drawn around individual bands and the relative intensity for the area within can be obtained by standard analyses by software such as ImageQuant. A similarly sized control box for background subtraction should be placed on an area of the gel that lacks obvious bands.

4. These assays can also be useful for characterization of ligand–RNA interactions by setting up reactions with a range of ligand concentrations. To account for subtle differences in loading, the relative intensity for the area within an individual box can be divided by the relative intensity for the area within a boxed region that encompasses the entire lane. If

the lower ligand concentrations and upper ligand concentrations are below and at ligand saturation, respectively, these experimental data may be used for estimation of EC_{50} values or estimates of cooperativity. For this type of analysis, multiple individual bands that display increased and decreased intensity in response to ligand interactions or conformational changes should be directly compared with one another. The easiest and most rapid method is to normalize each box series to the boxed band with the highest and lowest intensity measurements. If these values are normalized to maximal and minimal values of 1 and 0, respectively, then multiple band series can be compared to one another despite potentially significant differences in their overall relative intensity. A highly recommended alternative to these standard types of data analyses is a very useful software program called SAFA (*S*emi-*A*utomated *F*ootprinting *A*nalysis) (23). The latter software package significantly shortens the gel quantification process while reducing systematic error introduced during data analysis.

4. Notes

1. While T7 RNA polymerase is commonly used for high yield production of RNA by in vitro transcription, other RNA polymerases such as SP6 can also be used. However, when other RNA polymerases are used in these reactions, their individual promoter preferences onto DNA templates should be incorporated into the DNA template.

2. Inorganic pyrophosphatase is a useful addition to in vitro transcription reactions in order to improve the yield of RNA. During transcription, pyrophosphates will be released into solution which can result in the chelation of Mg^{2+} and subsequently the reduction of RNA polymerase function. Inorganic pyrophosphatase will reduce the accumulation of pyrophosphates.

3. Use of a high pH CHES buffer (5×: 25 mM $MgCl_2$, 125 mM CHES, pH 9, 15 mM DTT) can aid in the removal of oligonucleotide structure near the 5′ portion of an RNA molecule, which can interfere with the ability of PNK to phosphorylate the 5′-termini. The 10× buffer supplied by New England Biolabs can be used when structure is not a problem.

4. Glycogen is an optional but useful addition during ethanol precipitation because it precipitates the RNA and allows for easy visualization of the pellet. It is inert and its presence should not affect subsequent reactions.

5. Radiolysis of labeled RNAs can be a source of background noise in probing reactions. We recommend storing 5′-radiolabeled RNA at <100 kcpm/μL to reduce this problem. 3′-terminus radiolabeled RNA could also be used as substrates for in-line probing, although methods of 3′-labeling are not discussed herein.

6. In-line probing can be also used to study magnesium-dependent folding pathways under high monovalent conditions. When employing in-line probing to investigate divalent ion-induced RNA folding pathways, MgCl$_2$ should be eliminated from the 2× in-line/SHAPE buffer and it is recommended to increase monovalent concentrations (see next note). The individual components of the 2× SHAPE buffer can also be significantly varied (13, 14). We typically perform SHAPE reactions with a final concentration of monovalent ions ranging from 0 to 200 mM and concentrations of magnesium ranging from 0 to 40 mM. Typically both in-line probing and SHAPE reactions are performed at pH 7.5–8.3 (14). However, our standard SHAPE and in-line probing reactions are conducted at pH 8.0 and 8.3, respectively.

7. If high monovalent ions do not disrupt the molecular interactions being studied, in-line probing reactions can be performed in the presence of 1–3 M monovalent cations. Under these conditions, MgCl$_2$ can be eliminated from the reactions and varied as desired with little effect on the general efficiency of backbone self-cleavage. Additionally, monovalent ions at high concentrations are likely to outcompete the loosely associated "atmosphere" of divalent ions (24, 25). At lower monovalent cation concentrations, variations of MgCl$_2$ in in-line probing reactions can lead to modest fluctuations in banding intensities for all bands within the individual lanes. High monovalent cation conditions have also been successfully used for SHAPE analysis of magnesium-induced folding of RNAs (21).

8. While we have found that a 65-mM NMIA solution (6.5 mM final concentration) is ideal for RNA constructs ranging from 100 to 300 nucleotides, NMIA concentration is a parameter that should be varied during troubleshooting (13, 14). For RNA constructs shorter than 100 nucleotides in length, the NMIA solution should be increased (e.g., 130 mM). For RNA constructs longer than 300 nucleotides, a 30-mM NMIA solution is likely to be preferable. If low signal is observed in the +NMIA lanes but an intense band representing full length product is seen, this is an indication of insufficient modification of the RNA and higher concentrations of NMIA should be tested. If low molecular weight intense bands are observed and little

to no full length product can be seen, this is an indication of excessive modification of the RNA and lower concentrations of NMIA should be tested (14).

9. Superscript III (Invitrogen) or other commercial reverse transcriptase enzymes can be used with similar results, although the reaction temperatures may need to be individually optimized.

10. A dust mask, gloves, and eye protection should be worn when handling powdered acrylamide due to the fact that acrylamide is extremely neurotoxic until polymerized.

11. Upon purchase, the outside of the glass plates should be permanently marked so that proper orientation of the plates can be maintained in all subsequent runs.

12. Formation of a white precipitate during in vitro transcription is indicative of the accumulation of high levels of pyrophosphates. This will lead to a reduction in transcription, therefore the reaction can be stopped prior to the full 2–3 h incubation if significant precipitation is observed. The optional addition of inorganic pyrophosphatase will prevent the formation of this precipitate and increase the RNA yield.

13. Alternatively, the transcription reaction can be stopped by the addition of phenol:chloroform:isoamyl alcohol – see methods from **Section 3.2 steps 2 and 3**. This method is recommended if the transcription reaction has been scaled up in volumes greater than 100 μL to increase the yield of RNA.

14. Size-exclusion chromatography can also be used to purify the RNA of interest if an FLPC and an appropriate gel filtration column is available. The Superdex 200 10/300 GL column (GE Healthcare) can be used successfully to purify RNA molecules ranging in size from 10 to 60 kDa while a Superdex 75 10/300 GL column will resolve molecules approximately sized between 3 and 70 kDa. After the transcription reaction has been resolved by size-exclusion chromatography, RNA-containing fractions should be pooled and concentrated (by either ethanol precipitation or centrifugation-based concentrator devices) (*see* **Section 3.2 step 3**).

15. For in-line probing, shorter incubation times result in fainter bands but longer incubation times result in higher background. A highly recommended discussion on the rates of spontaneous cleavage of RNA linkages and effects of pH and metal ions can be found elsewhere (12, 20).

16. While bands on an in-line probing gel are typically indicative of flexible RNA linkages, certain bands may result from

nucleotides that are positioned such that the 2′hydroxyl and oxyanion leaving group most closely approximate the in-line conformation required for efficient phosphodiester scission (12).

17. Although the volume at this point of the SHAPE protocol is 18 μL, the final volume of the reaction will be a total of 20 μL so this should be taken into account when calculating the amount of ligand to add.

18. Prior to addition of the 2× in-line/SHAPE buffer, it is recommended to resuspend the RNA in a low ionic strength buffer such as 0.5× TE, heating to 95°C for 2 min, and snap cooling by placing on ice for 2 min (14). However, this step may be unnecessary, depending on the particular RNA molecule being investigated.

19. While 37°C is a useful temperature for many applications of SHAPE technology, this method can be used at temperatures ranging from 20 to 75°C (14, 26). For this method to be used at different temperatures, the NMIA modification step must be adapted. The NMIA should be incubated with the RNA for 5 half lives of hydrolysis. The half life of hydrolysis for NMIA at 37°C is ~8.3 min; therefore, at 37°C the NMIA modification step is incubated for ~45 min. However, the half-life of NMIA hydrolysis changes at different temperatures and can be calculated using the equation: half life (minutes) = 360∗exp [−0.102∗temperature (°C)].

References

1. Winkler, W. C., Breaker, R. R. (2005) Regulation of bacterial gene expression by riboswitches. *Annu Rev Microbiol* 59, 487–517.

2. Schwalbe, H., Buck, J., Furtig, B., Noeske, J., Wohnert, J. (2007) Structures of RNA switches: insight into molecular recognition and tertiary structure. *Angew Chem Int Ed Engl* 46, 1212–1219.

3. Wakeman, C. A., Winkler, W. C., Dann, C. E. III. (2007) Structural features of metabolite-sensing riboswitches. *Trends Biochem Sci* 32, 415–424.

4. Edwards, T. E., Klein, D. J., Ferré-D'Amaré, A. R. (2007) Riboswitches: small-molecule recognition by gene regulatory RNAs. *Curr Opin Struct Biol* 17, 273–279.

5. Gilbert, S. D., Batey, R. T. (2006) Riboswitches: fold and function. *Chem Biol* 13, 805–807.

6. Winkler, W., Nahvi, A., Breaker, R. R. (2002) Thiamine derivatives bind messenger RNAs directly to regulate bacterial gene expression. *Nature* 419, 952–956.

7. Serganov, A., Polonskaia, A., Phan, A. T., Breaker, R. R., Patel, D. J. (2006) Structural basis for gene regulation by a thiamine pyrophosphate-sensing riboswitch. *Nature* 441, 1176–1171.

8. Ehresmann, C., Baudin, F., Mougel, M., Romby, P., Ebel, J. P., Ehresmann, B. (1987) Probing the structure of RNAs in solution. *Nucleic Acids Res* 15, 9109–9028.

9. Werner, C., Krebs, B., Keith, G., Dirheimer, G. (1976) Specific cleavages of pure tRNAs by plumbous ions. *Biochim Biophys Acta* 432, 161–175.

10. Brunel, C., Romby, P. (2000) Probing RNA structure and RNA-ligand complexes with chemical probes. *Methods Enzymol* 318, 3–21.

11. Ryder, S. P., Strobel, S. A. (1999) Nucleotide analog interference mapping. *Methods* 18, 38–50.

12. Soukup, G. A., Breaker, R. R. (1999) Relationship between internucleotide linkage geometry and the stability of RNA. *RNA* 5, 1308–1325.

13. Merino, E. J., Wilkinson, K. A., Coughlan, J. L., Weeks, K. M. (2005) RNA structure analysis at single nucleotide resolution by selective 2′-hydroxyl acylation and primer extension (SHAPE). *J Am Chem Soc* 127, 4223–4231.

14. Wilkinson, K. A., Merino, E. J., Weeks, K. M. (2006) Selective 2′-hydroxyl acylation analyzed by primer extension (SHAPE): quantitative RNA structure analysis at single nucleotide resolution. *Nature Protoc* 1, 1610–1616.

15. Nahvi, A., Sudarsan, N., Ebert, M. S., Zou, X., Brown, K. L., Breaker. R. R. (2002) Genetic control by a metabolite-binding mRNA. *Chem Biol* 9, 1043.

16. Westheimer, F. H. (1968) Pseudo-rotation in the hydrolysis of phosphate esters. *Acc Chem Res* 1, 70–78.

17. Usher, D. A. (1969) On the mechanism of ribonuclease action. *Proc Natl Acad Sci USA* 62, 661–627.

18. Usher, D. A., McHale, A. H. (1976) Hydrolytic stability of helical RNA: a selective advantage for the natural 3′, 5′-bond. *Proc Natl Acad Sci USA* 73, 1149–1153.

19. Dock-Bregeon, A. C., Moras, D. (1987) Conformational changes and dynamics of tRNAs: evidence from hydrolysis patterns. *Cold Spring Harb Symp Quant Biol* 52, 113–121.

20. Li, Y., Breaker, R. R. (1999) Kinetics of RNA degradation by specific base catalysis of transesterification involving the 2′-hydroxyl group. *J Am Chem Soc* 121, 5364–5372.

21. Dann, C. E. III, Wakeman, C. A., Sieling, C. L., Baker, S. C., Irnov, I., Winkler, W. C. (2007) Structure and mechanism of a metal-sensing regulatory RNA. *Cell* 130, 878–892.

22. Mortimor, S. A., Weeks, K. M. (2007) A fast-acting reagent for accurate analysis of RNA secondary and tertiary structure by SHAPE chemistry. *J Am Chem Soc* 129, 4144–4145.

23. Das, R., Laederach, A., Pearlman, S. M., Herschlag, D., Altman, R. B. (2005) SAFA: a semi-automated fooprinting analysis software for high-throughput quantification of nucleic acid footprinting experiments. *RNA* 11, 344–354.

24. Das, R., Travers, K. J., Bai, Y., Herschlag, D. (2005) Determining the Mg^{2+} stoichiometry for folding an RNA metal ion core. *J Am Chem Soc* 127, 8272–8273.

25. Draper, D. E., Grilley, D., Soto, A. M. (2005) Ions and RNA folding. *Annu Rev Biophys Biomol Struct* 34, 221–243.

26. Wilkinson, K. A., Merino, E. J., Weeks, K. M. (2005) RNA SHAPE chemistry reveals non-hierarchical interactions dominate equilibrium structural transitions in tRNA[Asp] transcripts. *J Am Chem Soc* 127, 4659–4667.

27. Winkler, W., Nahvi, A., Breaker, R. R. (2002) Thiamine derivatives bind messenger RNAs directly to regulate bacterial gene expression. *Nature* 419, 952–956.