Probing RNA Structure and Metal-Binding Sites Using Terbium(III) Footprinting

BASIC PROTOCOL

Metal ions play a crucial role in RNA tertiary structure folding by neutralizing and bridging the negatively charged phosphoribose backbone. A folded RNA is stabilized by specific and nonspecific interactions with metal ions. The current unit describes the use of the lanthanide metal ion terbium(III) as a probe of both RNA structure and high-affinity metal-binding sites. Thus, it complements *UNIT 6.3*, since both the lead (Pb^{2+}) and terbium (Tb^{3+}) aqueous hydroxo complexes cleave the RNA backbone in a similar fashion. Specifically, terbium(III) binds to the same sites on RNA as magnesium(II), but with two to four orders of magnitude higher affinity. Low (micromolar) concentrations of terbium(III) will therefore displace magnesium and result in hydrolytic backbone cleavage at high-affinity magnesium-binding sites. At high (millimolar) concentrations of terbium(III), cleavage occurs in a largely sequence-independent manner, preferentially cutting single-stranded or non-Watson-Crick base-paired regions. The aqueous terbium(III) complex has a near-neutral pK_a (~7.9), which enables it to hydrolyze the RNA backbone around physiological pH. This metal ion–promoted RNA backbone cleavage occurs via deprotonation of the 2′-hydroxyl group and nucleophilic attack of the resulting oxyanion on the adjacent 3′,5′-phosphodiester bond to form 2′,3′-cyclic phosphate and 5′-hydroxyl termini (Figure 6.8.1). Since cleavage is dependent on terbium(III) being able to access the 2′-hydroxyl group, the terbium(III) cleavage pattern can be used to probe the secondary and tertiary structure within an RNA molecule.

In the protocol below, which is a standard procedure for performing terbium(III)-mediated footprinting of an RNA molecule, RNA that has been end-labeled with ^{32}P at either the 5′ or 3′ end is prefolded in an appropriate buffer including divalent cations (generally magnesium). Varying concentrations of terbium(III) are added to the RNA mixture to initiate the strand-scission reaction, and the mixture is incubated 30 min to 2 hr. The reaction is quenched, the cleaved RNA is separated on a denaturing polyacrylamide gel, and the cleavage pattern is visualized by exposure of the gel to a phosphor imager screen. Partial alkaline hydrolysis and ribonuclease (RNase) T1 digestion reactions of the radiolabeled RNA are analyzed alongside the terbium(III)-mediated cleavage reaction to accurately locate the cleavage sites (see *UNIT 6.1*). The intensities of cleaved bands are quantified and a footprint of the overall RNA molecule is obtained.

CAUTION: When working with radioactive materials, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in an appropriately designated area, following the guidelines provided by the local radiation safety officer.

NOTE: As for experiments with RNA in general, care must be taken to avoid introducing ribonucleases (RNases) into the samples. This can be avoided by wearing gloves to avoid skin contact when handling samples and solutions; by using nuclease-free, sterilized pipet tips, sample tubes, and other disposable plasticware; by preparing all solutions from highest-purity (e.g., molecular-biology grade) components in double-deionized water (18 MΩ conductivity); and by filter-sterilizing (through a 0.22-μm filter) or autoclaving all solutions. Although less desirable, treatment with diethylpyrocarbonate (DEPC; *APPENDIX 2A*) may be used to block background nuclease activities.

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Materials

RNA, gel-purified (*UNIT 6.1*), labeled at 5′ or 3′ end with ^{32}P (conc. ≥50,000 cpm/μL; sp. act. ≥150,000 cpm/pmol)
Appropriate buffers to fold RNA (usually Tris, MES, and/or HEPES buffer of desired pH; see Critical Parameters for discussion of optimization)
1 M $MgCl_2$ (*APPENDIX 2A*)
100 mM $TbCl_3$ (see recipe)
0.5 M EDTA, pH 8.0 (*APPENDIX 2A*)
3 M sodium acetate (*APPENDIX 2A*)
80% and 100% (v/v) ethanol
Urea loading buffer (*APPENDIX 2A*)

Heating block
Water bath at optimized incubation temperature (25° to 45°C)
Phosphor screens and phosphor imager with appropriate software (e.g., PhosphorImager Storm 840 with ImageQuant software; Molecular Dynamics)

Additional reagents and equipment for partial alkaline hydrolysis and RNase T1 digestion of RNA (*UNIT 6.1*, Support Protocol 3), and denaturing polyacrylamide gel electrophoresis (*APPENDIX 3B*)

Prepare reaction mixture and terbium(III) dilution series

1. Mix the desired amount of end-labeled RNA with appropriate buffer. Prepare a single pool containing 250,000 to 500,000 cpm (typically 0.5 to 2 pmol) RNA per aliquot, with a total volume that will be sufficient for duplicate 8-μL aliquots at each terbium(III) concentration (see steps 4 and 5). Denature 2 min in a 90°C heating block.

   A micromolar background of unlabeled RNA can be added to reduce nonspecific cleavage at low terbium(III) concentrations and to facilitate subsequent ethanol precipitation.

2. Slowly renature the RNA by incubating mixture at an optimized temperature between 25° and 45°C (see Critical Parameters) for ~10 min.

3. Add $MgCl_2$ from 1 M or other appropriately diluted stock solution to obtain desired Mg^{2+} concentration (see Critical Parameters) and continue to incubate at appropriate temperature for an additional 5 min.

4. Make a serial set of $TbCl_3$ dilutions in water from the 0.1 M $TbCl_3$ stock solution, ranging from micromolar to millimolar concentrations, calculated at 5× the final reaction concentration (see step 5).

   The $TbCl_3$ dilutions in water should be made immediately prior to use. A serial set of dilutions is recommended to ensure consistency in cleavage band intensity between gel lanes. Use a fresh aliquot of 0.1 M $TbCl_3$ stock solution each time. Final $TbCl_3$ concentrations used in the cleavage reactions should be optimized together with other experimental conditions for the specific RNA and experimental goal (see Critical Parameters).

Run reaction

5. Initiate terbium(III)-mediated cleavage reactions by combining 8 μL RNA (step 3) with 2 μL of each $TbCl_3$ dilution (step 4) to achieve the desired final Tb^{3+} concentrations (typically 0 to 5 mM). Perform each concentration in duplicate. Continue to incubate at the optimized temperature (25° to 45°C) for an optimized length of time (30 min to 2 hr; see Critical Parameters).

   Incubation times between 30 min and 2 hr are sufficient to generate a partial digestion pattern of end-labeled RNA under single hit conditions. Avoid more extended incubation times to prevent secondary hits (see Critical Parameters).

6. Terminate the cleavage reaction by adding 0.5 M EDTA, pH 8.0, to a final concentration of 50 mM (or at least a 2-fold excess over the total concentration of multivalent metal ions).

Collect RNA and analyze cleavage patterns

7. Add 3 M sodium acetate to the reaction to final concentration of 0.3 M, then add 2 to 2.5 vol of 100% ethanol and incubate overnight −20°C to precipitate the RNA. Centrifuge 30 min at 12,000 × *g*, 4°C. Decant supernatant, wash pellet with 80% ethanol, decant supernatant, and dry RNA in a Speedvac evaporator. Redissolve samples in 10 to 20 µL of urea loading buffer.

8. Using separate samples of the same end-labeled RNA, perform partial alkaline hydrolysis and RNase T1 digestion reactions (see *UNIT 6.1*, Support Protocol 3). Add 10 µL urea loading buffer to an equal volume (10 µL) of the partial hydrolysis reaction and the T1 digestion, and load each directly onto the gel.

9. Place all samples in a heating block at 90°C for 5 min, then snap cool in ice water. Analyze the cleavage products on a high-resolution denaturing (8 M urea) polyacrylamide gel (*APPENDIX 3B*), using the partial alkaline hydrolysis and RNase T1 digestion reactions as sequencing ladders to identify the specific terbium(III) cleavage products at nucleotide resolution.

   The authors use a 15% wedged gel, run at a constant power of 80 W, for separating an 80-mer RNA. Identical samples can be loaded at different times on the same gel to resolve different regions of the RNA.

10. Expose the gel to a phosphor imager screen.

    This can take several hours to overnight, depending on the level of radioactivity of the bands on the gel.

11. Scan the phosphor screen and quantify the full-length RNA and cleavage-product bands using a volume count method. At every Tb^{3+} concentration, calculate a normalized extent of cleavage (Π) by substituting the peak intensities in the equation:

$$\Pi = \frac{\left(\dfrac{\text{band intensity at nucleotide } x}{\sum_i \text{band intensity at nucleotide } i}\right)_{y\,[Tb^{3+}]}}{\left(\dfrac{\text{band intensity at nucleotide } x}{\sum_i \text{band intensity at nucleotide } i}\right)_{0\text{ mM }[Tb^{3+}]}}$$

   where *y* is the terbium(III) concentration in a particular cleavage reaction, *x* is the analyzed nucleotide position of the RNA, and "0 mM [Tb^{3+}]" signifies a control reaction containing no terbium (III), incubated in the same fashion as reactions containing terbium(III).

   A Π *value of ≥2 indicates significant cleavage over background degradation. By dividing the ratio of a single band intensity over total RNA in the presence of terbium(III) by the ratio of a single band intensity over total RNA in the absence of terbium(III), one normalizes for the effect of nonspecific background degradation.*

REAGENTS AND SOLUTIONS

*Use double-deionized nuclease-free water (18 MΩ conductivity) in all recipes and protocol steps (also see General Considerations for Working with RNA in **APPENDIX 2A**). To eliminate traces of RNases on glassware, rinse in RNase-free water (**APPENDIX 2A**) and autoclave or bake 2 hr at 150°C. For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.*

Sodium cacodylate buffer, 5 mM (pH 5.5)

Prepare 15.9 g/L sodium cacodylate (100 mM final). Adjust to pH 7 with NaOH or cacodylic acid. Dilute 100 mM stock solution to 5 mM, sterilize by filtering with a 0.22-µm filter, and store up to several months at room temperature.

CAUTION: *Be careful when handling the toxic cacodylate.*

Terbium(III) chloride

Dissolve 3.73 g solid $TbCl_3 \cdot 6H_2O$ (Aldrich; 99.9%) in 100 ml of 5 mM sodium cacodylate buffer (see recipe) to give a final concentration of 0.1 M. Filter through 0.22-µm filters into 1-mL aliquots and store up to 1 year at −20°C.

The 0.1 M $TbCl_3$ stock solution is prepared in a 5 mM sodium cacodylate buffer at pH 5.5 to prevent precipitation of terbium(III) hydroxide, which occurs at higher pH.

COMMENTARY

Background Information

Tertiary structure formation in RNA is highly influenced by metal ions, particularly divalents (Pyle, 2002). The lanthanide metal ion terbium(III) has proven to be a powerful probe of tertiary structure, as well as a tool for mapping metal-binding sites in RNA. Terbium(III) binds to sites on RNA in a manner similar to Mg^{2+}, with an affinity two to four orders of magnitude higher (Walter et al., 2000). Terbium(III) has the ability to substitute for magnesium because hydrated Tb^{3+} ions have an ionic radius (0.92 Å) similar to that of hydrated Mg^{2+} (0.72 Å), and because terbium(III) and magnesium share the same preference for coordination to oxygen ligands (Saito and Suga, 2002). There are several classes of metal-binding sites found in RNA, ranging from diffuse to site-specific, and involving both inner- and outer-sphere coordination to the RNA. The most common ligands on the RNA are the nonbridging phosphate oxygens, the purine N7 positions, the base keto groups, and the ribose 2′-hydroxyls. Once bound to RNA, terbium(III) cleaves the phosphodiester backbone by abstracting the 2′-hydroxyl of a nearby nucleotide. This allows for nucleophilic attack of the resulting 2′-oxyanion on the juxtaposed phosphodiester bond, leading to strand cleavage (Ciesiolka et al., 1989; Matsumura and Komiyama, 1997; Figure 6.8.1). Tb^{3+} can effectively catalyze cleavage of an RNA backbone at physiological pH due to its lower pK_a(~7.9) compared to that of Mg^{2+} (Sigel et al., 2000).

By varying the concentration of Tb^{3+} used in the cleavage reaction, it is possible to obtain a map of metal-binding sites and a footprint of secondary and tertiary structure. Micromolar concentrations of Tb^{3+} bind to high-affinity metal binding sites within a folded RNA, leading to few specific cleavage sites. By contrast, millimolar concentrations of Tb^{3+} produce a footprinting pattern of solvent-accessible regions, mainly cleaving the RNA backbone in a sequence-independent manner. Under these conditions, the backbone is preferentially cut in single-stranded and non-Watson-Crick base-paired regions, possibly due to the better accessibility of ligands such as N7 positions of purines and the π-electron systems of all nucleobases (Walter et al., 2000). Therefore, it is critical to perform the cleavage reactions over a wide range of Tb^{3+} concentrations in order to acquire information on both high-affinity metal binding and secondary and tertiary structure folding.

Terbium(III)-mediated footprinting and other properties of this lanthanide ion have provided for a versatile probe of RNA structure, function, and metal-ion binding. For example, micromolar concentrations of terbium(III) not only report a metal binding site in loop B of the hairpin ribozyme through backbone cleavage, but also permit the analysis of metal-binding affinity and kinetics through sensitized luminescence, and reversibly inhibit the ribozyme's catalytic activity by competing for a crucial, yet nonselective, cation-binding site (Walter et al., 2000). Millimolar terbium(III) concentrations,

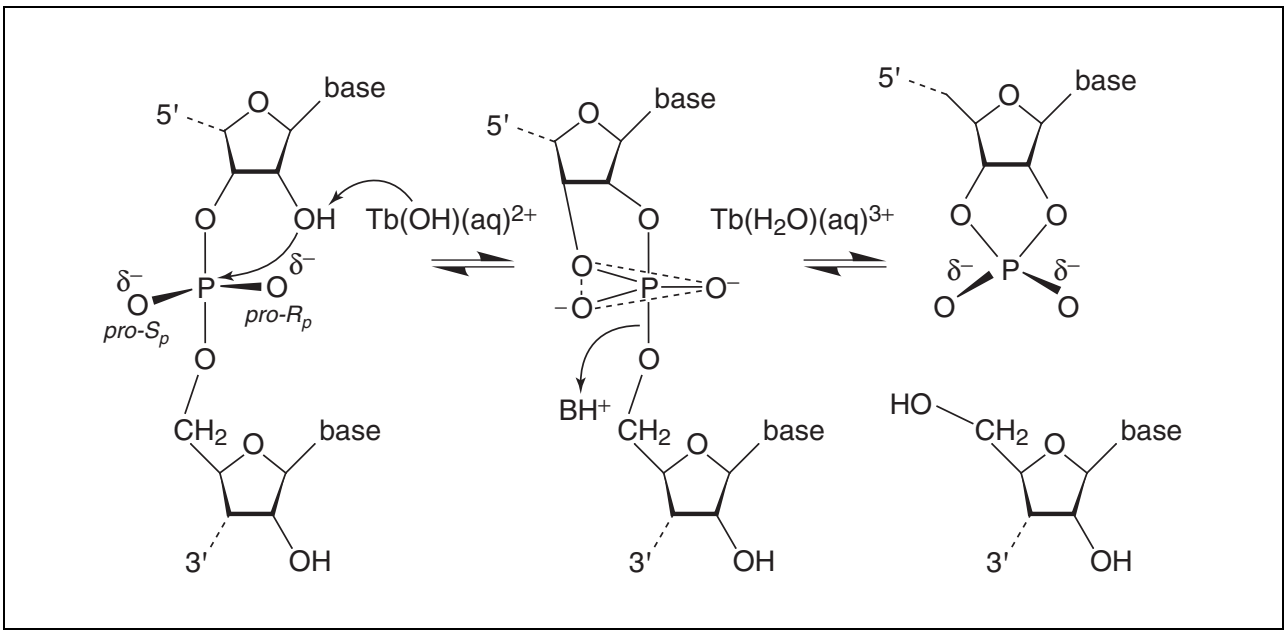

Figure 6.8.1 Terbium(III)-mediated RNA backbone cleavage. The aqueous terbium(III) hydroxo complex $Tb(OH)(aq)^{2+}$ deprotonates the 2′-hydroxyl group, allowing for nucleophilic attack of the resulting oxyanion on the adjacent phosphodiester bond. This leads to formation of a trigonal-bipyramidal transition state (center), which, upon protonation by a general acid (BH^+), breaks down to form 2′,3′-cyclic phosphate and a 5′-hydroxyl terminus.

in contrast, footprint the hairpin ribozyme's secondary and tertiary structure and reveal a solvent-protected core similar to that observed in hydroxyl radical footprinting (Walter et al., 2000; also see Figure 6.8.2). At low Tb^{3+} concentrations, cleavage of human $tRNA^{Lys,3}$ is restricted to nucleotides that were previously identified from X-ray crystallography as specific metal-binding pockets (Hargittai and Musier-Forsyth, 2000). The use of higher Tb^{3+} concentrations resulted in an overall footprint of the L-shaped tRNA structure, showing increased cleavage in the loop regions (D and anticodon loop; Hargittai and Musier-Forsyth, 2000). HIV nucleocapsid protein could then be shown to result in the disruption of the tRNA's metal-binding pockets and, at higher concentrations, to induce subtle structural changes in, for example, the tRNA acceptor-TψC stem minihelix (Hargittai et al., 2001). Other RNAs that have similarly been studied by terbium(III)-mediated footprinting include the hammerhead (Feig et al., 1999), aminoacyltransferase (Flynn-Charlebois et al., 2001; Vaidya and Suga, 2001), RNase P (Kaye et al., 2002), and group II intron ribozymes (Sigel et al., 2000).

Critical Parameters

Tb^{3+} can be a very useful chemical probe of metal binding and tertiary structure folding in RNA. However, there are several parameters that are important to consider in order to obtain a reliable and reproducible RNA footprint. Prefolding the RNA under the correct buffer conditions and magnesium concentrations will ensure conformational homogeneity. When optimizing these components, the pH of the cleavage reaction should be kept near physiological pH (7.0 to 7.5), allowing for the accumulation of the cleavage-active $Tb(OH)(aq)^{2+}$ species (Walter et al., 2000). At a pH above 7.5, Tb^{3+} increasingly forms insoluble polynuclear hydroxo aggregates (Baes and Mesmer, 1976; Matsumura and Komiyama, 1997), which should be avoided. Precipitation of such insoluble polynuclear species lowers the concentration of the cleavage-active mononuclear species, $Tb(OH)(aq)^{2+}$, and potentially co-precipitates the RNA. The temperature at which the cleavage reaction is performed must be experimentally determined. Higher temperatures result in faster cleavage rates but also increase the amount of background degradation. Therefore, typical reaction temperatures range from 25° to 45°C.

Since Tb^{3+} has the ability to displace Mg^{2+} in high-affinity binding pockets while also footprinting solvent-accessible regions, identifying a metal-binding site must be done with care. An effective means of locating a high-affinity binding site is to first decrease the Tb^{3+}

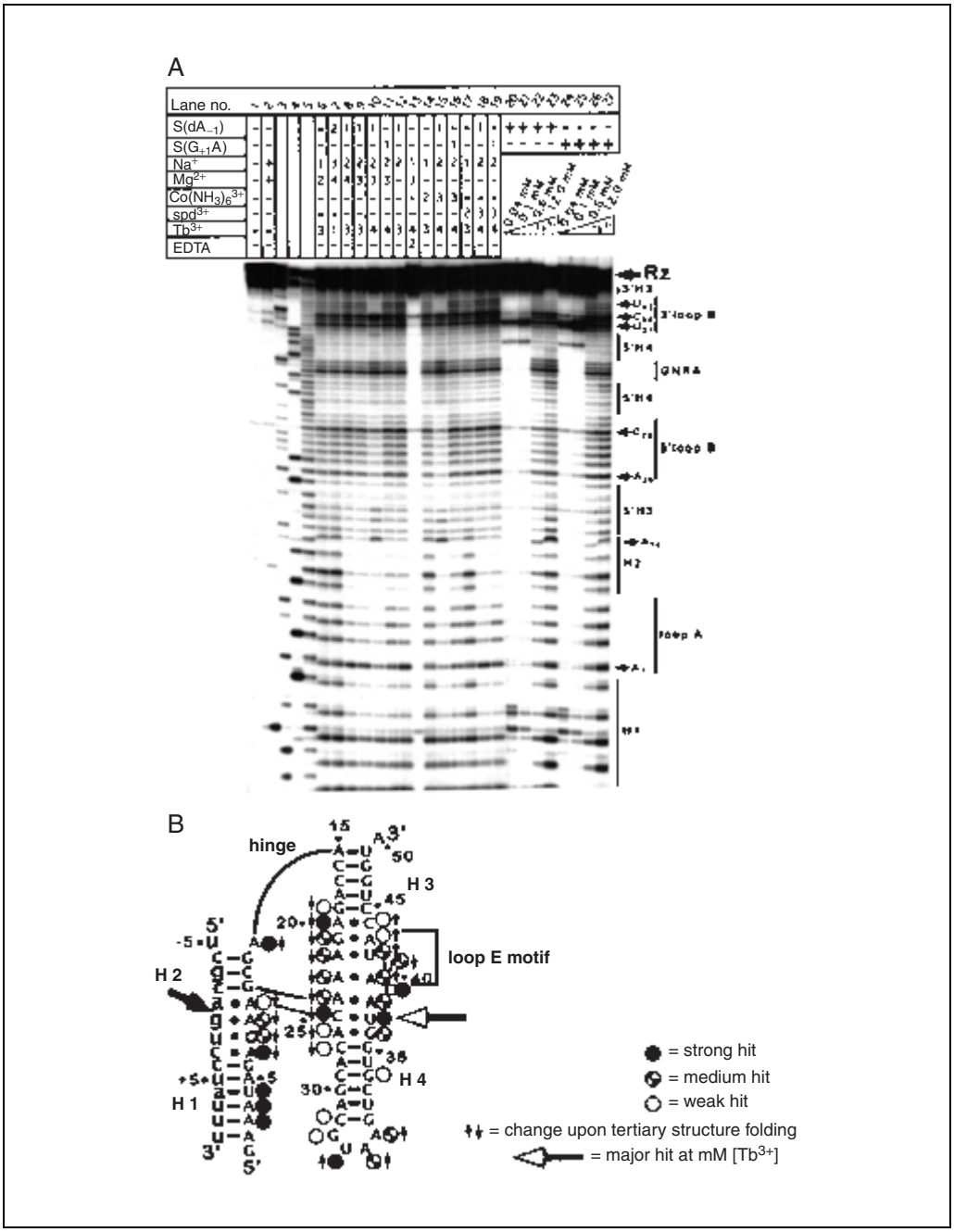

Figure 6.8.2 Terbium(III)-mediated footprinting of the hairpin ribozyme (Walter et al., 2000). (**A**) Footprint of 5′-^{32}P-labeled hairpin ribozyme (Rz) after incubation with terbium(III) for 2 hr at 25°C. Lanes 1 and 2, controls; lanes 3 and 4, sequencing ladders with RNase U2 (cutting after A) and RNase T1 (cutting after G); lane 5, partial alkali hydrolysis; lanes 6 to 19, order-of-addition experiments (numbers indicate order) with a 100-fold excess (500 nM) of noncleavable substrate analog S(dA$_{-1}$) or S(G$_{+1}$A), 100 mM Na^+, 12 mM Mg^{2+}, 12 mM $Co(NH_3)_6^{3+}$, and 12 mM spermidine (spd^{3+}), 12 mM Tb^{3+}, and 120 mM EDTA as chelator; lanes 20 to 27, incubation of 10 μM ribozyme-substrate complex, containing trace amounts of radiolabeled ribozyme, in the presence of 12 mM Mg^{2+} with varying concentrations of Tb^{3+}, as indicated, and with noncleavable substrate analog, either S(dA$_{-1}$) or S(G$_{+1}$A). (**B**) Superposition of the observed backbone cleavage sites at 12 mM terbium(III) onto a schematic of the hairpin ribozyme. Strong, medium, and weak hits, together with changes caused by tertiary-structure formation, are indicated at the nucleotide 3′ to which cleavage occurs, as deduced from quantitation of lanes 8 (footprint of secondary structure; Tb^{3+} added before magnesium, thus preventing tertiary structure folding) and 10 (footprint of tertiary structure; magnesium added before Tb^{3+}) in (A). The only major hit at micromolar concentrations of terbium(III) is 3′ to U37. Reproduced from Walter et al. (2000) with permission from Elsevier.

concentration until a very narrow cleavage pattern is observed (typically at 10 to 100 μM Tb^{3+}), and then to perform a competition experiment with increasing concentrations of Mg^{2+}. This can be done as in the Basic Protocol, but the concentration of Mg^{2+} is increased against a constant Tb^{3+} concentration. The residual terbium(III)-mediated cleavage bands should diminish as the Mg^{2+} concentration is increased.

The optimal Tb^{3+} concentration(s) to use for structure probing are also an important consideration and must be determined for each individual RNA. The trivalent terbium(III) has been shown to induce slight perturbations in the RNA structure (Hargittai and Musier-Forsyth, 2000), but careful titration will reveal the optimal terbium(III):RNA ratio needed for detecting secondary and tertiary structure features in a given RNA molecule.

Anticipated Results

A typical result involving both terbium(III)-mediated structure probing and metal-binding-site detection is shown in Figure 6.8.2 for the hairpin ribozyme (Walter et al., 2000). The cleavage pattern on such a denaturing polyacrylamide gel normally shows many bands of varying intensities. These varying intensities (or extents of cleavage) at each nucleotide position relate to the structure of the RNA. The extent of total cleavage should generally be no more than 20% of the uncleaved or full-length band. This ensures that the RNA undergoes a single hit and minimizes the presence of products resulting from multiple cleavage events. The normalized extent of cleavage (Π) is best quantified according to a modified form of the equation used in nucleotide analog interference mapping (NAIM) analysis (Ryder and Strobel, 1999), as given above (see Basic Protocol, step 11).

It is important to note that terbium(III) footprinting may not reveal all high-affinity metal binding sites due to the geometrical requirement of Tb^{3+} accessibility to the 2′-hydroxyl group on the ribose (Figure 6.8.1). The rate and extent of cleavage depend on distance between the terbium(III) and the 2′-hydroxyl of the nearby nucleotide, which is unfavorable in a standard A-type RNA helix. Therefore, strong metal sites that occur in RNA helical regions may go undetected by Tb^{3+} cleavage (Sigel et al., 2000).

Time Considerations

The procedure outlined in the Basic Protocol provides an easy and sensitive means of probing a wide variety of RNA structures. All procedures in this unit can be completed in 2 to 3 days. The most time-consuming aspect involved is the end labeling and careful gel purification of the RNA (*UNIT 6.1*). The cleavage reactions themselves take no more than 2 hr and are followed by gel electrophoresis (3 to 5 hr) and phosphorimager analysis (6 to 24 hr).

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