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METHOD

RNA footprinting analysis using ion pair reverse phase liquid chromatography

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ABSTRACT

Hydroxyl radical footprinting is a powerful technique often employed in characterization of the tertiary interactions between proteins and nucleic acids. Following the generation of a nucleic acid "ladder" either by chemical or enzymatic reactions, the radiolabeled products are traditionally separated by denaturing gel electrophoresis and further quantified by phosphorimaging techniques. Here we report the use of ion pair reverse phase liquid chromatography to analyze the products of an RNA footprinting reaction using fluorescently labeled RNA molecules. This technique offers several advantages over existing procedures, including rapid analysis, automation, and direct quantification of the cleavage products without the need to employ radiolabeling. To illustrate the resolving power of this technique, we have analyzed the products of base hydrolysis, generated from a fluorescently labeled RNA molecule and have subsequently used this method to define the solvent accessibility of the substrate strand as it docks with the hairpin ribozyme.

Keywords: chromatography; hairpin ribozyme; hydroxyl radical; reverse-phase; RNA footprinting

INTRODUCTION

RNA footprinting is a powerful technique that is often employed to obtain RNA structural information in solution in the presence and absence of ligands. This technique provides information on the solvent accessibility within RNA molecules and can therefore be used to analyze tertiary interactions of small RNA structures (Celander & Cech, 1991) and RNA–protein interactions (Weeks & Cech, 1995). To differentiate between the internal and external regions of the folded RNA molecules, the solvent accessibility of the C4′ position of the ribose moiety can be monitored by the addition of an Fe(II)–EDTA complex together with hydrogen peroxide to the RNA in solution (Latham & Cech, 1989). The hydroxyl radicals generated primarily attack the C4′ position of the sugar resulting in cleavage of the phosphodiester bond (Wu et al., 1983). Following the generation of the nucleic acid ladder, the radiolabeled products are traditionally separated by gel electrophoresis and subjected to phosphorimaging techniques.

Here we report the use of ion pair reverse phase liquid chromatography (IP RP HPLC) to analyze the products of the footprinting reaction using fluorescently labeled RNA. This technique offers the advantages of rapid analysis, automation, and direct quantification without the need for radiolabeled materials. To illustrate the high resolution obtained using this technique, we demonstrate the analysis of a base hydrolysis "ladder" generated from a fluorescently labeled RNA molecule. The ability to analyze hydroxyl radical footprinting reactions using IP RP HPLC is also demonstrated by probing the solvent accessibility of the substrate strand as it docks in the hairpin ribozyme complex.

The hairpin ribozyme is a small catalytic RNA that performs a reversible self-cleavage reaction. In vivo the hairpin ribozyme is produced as an intermediate during the course of rolling circle replication in the tobacco ringspot virus (reviewed by Fedor, 2000). The minimal component required for catalytic function in vitro includes four short helices and two internal loop regions, A and B (see Fig. 1). The ribozyme catalyzes the cleavage at a specific site within loop A, generating

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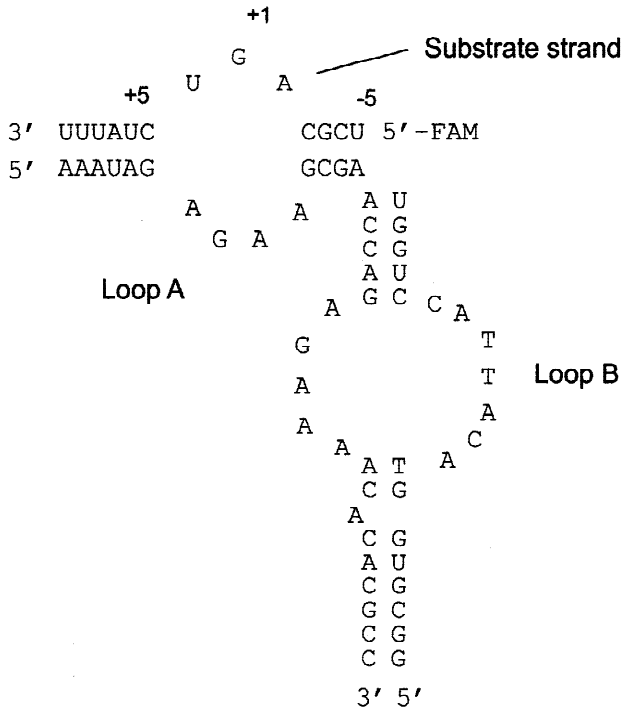

FIGURE 1. The hairpin ribozyme construct used in the footprinting analysis. A synthetic two-piece ribozyme–substrate complex was used in which the substrate strand was fluorescently labeled at the 5′ end with fluorescein.

products with a 5′-hydroxyl and 2′-3′-cyclic phosphate termini. Metal ions play a passive role in the hairpin ribozyme reaction; however, they are required to form the catalytically active structure that involves the docking of loop A and loop B (Hampel & Cowan, 1997; Nesbitt et al., 1997; Young et al., 1997). Recent crystallographic information of the hairpin ribozyme–inhibitor complex shows the active site of the natural RNA results from the docking of the stems of the two irregular helices A and B (Rupert & Ferré-D'Amaré, 2001). The crystal structure also reveals that the base of the ribonucleotide preceding the cleavage site is stacked within stem A and the following essential guanine (g + 1) is extruded from stem A into a complementary pocket in the minor groove of stem B. Metal ions were also shown to be absent from the active site. The crystal structure is consistent with previous results obtained using hydroxyl radical footprinting (Hampel et al., 1998). These results show that a solvent-inaccessible core is formed upon folding of the active ribozyme complex in the presence of polyvalent cations.

Hydroxyl radical footprinting experiments were performed in both the presence and absence of $Co(NH_3)_6^{3+}$ and the cleavage products were analyzed using IP RP HPLC. Protection was observed in the substrate strand in the docked ribozyme complex at positions a − 1 and u + 2, consistent with the recent crystallographic structure of the hairpin ribozyme complex (Rupert & Ferré-D'Amaré, 2001) and a previous footprinting analysis (Hampel et al., 1998).

RESULTS AND DISCUSSION

Separation conditions of fluorescently labeled RNA

The use of Tetrabutylammonium bromide (as the ion pairing reagent) with DNAsep® chromatography is essential for the size-dependent separation of fluorescently labeled RNA. This regime removes the influence of the hydrophobic fluorescent group and sequence specific effects. Figure 2A shows the chromatogram generated by base-catalyzed hydrolysis of the fluorescently labeled substrate strand of the hairpin ribozyme. The substrate strand was fluorescently labeled at the 5′ end with fluorescein. No cleavage is seen at the a − 1 position that contains a 2′-*O*-methyl group that is resistant to base-catalyzed hydrolysis. Figure 2B shows the chromatogram generated by base-catalyzed hydro-

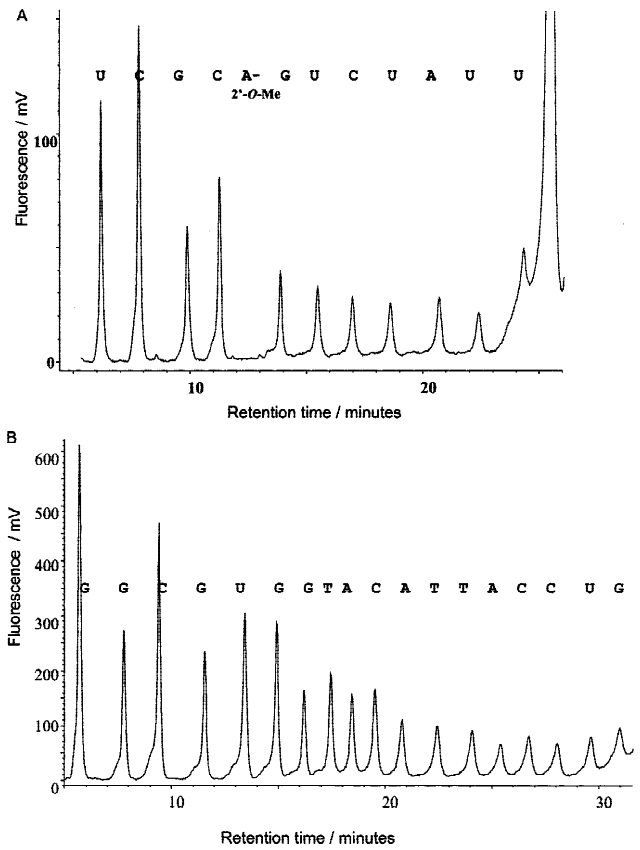

FIGURE 2. Base-catalyzed hydrolysis of fluorescently labeled RNA. **A:** Chromatogram generated from the base catalyzed hydrolysis of the substrate strand, analyzed using IP RP HPLC. No cleavage is seen at the (a − 1) position, which contains a 2′-*O*-methyl group that is resistant to base-catalyzed hydrolysis. **B:** Chromatogram generated from the base-catalyzed hydrolysis of the 5′-loop B RNA fluorescently labeled at the 5′ end with hexachlorofluorescein.

lysis of the fluorescently labeled 5′-loop B RNA. The 5′-loop B RNA was fluorescently labeled at the 5′ end with hexachlorofluorescein. The separation of the base-catalyzed cleavage products allows the alignment of the hydroxyl radical-generated cleavage products and demonstrates the high resolution obtained using IP RP HPLC. Subsequent to treatment with acid, following base hydrolysis, numerous additional fragments were observed during chromatography. This further demonstrates the high resolution capability of the DNAsep in the separation of the 5′-3′-cyclic phosphate intermediates and 3′-phosphate products. Using this technique, it is possible to achieve base pair resolution using oligonucleotides up to 60 nt in length.

Solvent accessibility of the substrate strand in the hairpin ribozyme

To analyze the solvent accessibility of the substrate strand in the folded ribozyme complex, hydroxyl radical footprinting reactions were carried out on the fluorescently labeled substrate strand. The results from hydroxyl radical footprinting of the fluorescently labeled substrate strand in the hairpin ribozyme complex in the presence and absence of $Co(NH_3)_6^{3+}$ are shown in Figure 3. Protection of the substrate was observed in the presence of $Co(NH_3)_6^{3+}$ spanning the substrate cleavage site (a − 1, g + 1, u + 2, and c + 3). These results are consistent with those obtained by Hampel et al. (1998), who observed protection at the c − 2, a − 1, g + 1, and u + 2 positions in the substrate strand, demonstrating that the C4′ atoms surrounding the cleavage site ribonucleotides are internalized upon folding of the hairpin ribozyme. These results are also in agreement with a tertiary structure model of the hairpin ribozyme (Earnshaw et al., 1997) and the recent crystallographic model of the hairpin ribozyme–inhibitor complex (Rupert & Ferré-D'Amaré, 2001).

Analyses of the accessibility of the C4′ positions of the ribonucleotides from the crystal structure coordinates were compared to the experimentally observed sites of protection in the substrate strand (see Fig. 3B). The protection seen at a − 1 and u + 2 is in agreement with this model. An illustration of the C4′ positions in the crystal structure of the hairpin ribozyme–inhibitor complex is shown in Figure 4. The sites of protection in the substrate strand determined by hydroxyl radical footprinting are highlighted on the model. No protection of the cleavage products was observed for the fluorescently labeled substrate strand in the absence of loop A and B RNA in the presence of $Co(NH_3)_6^{3+}$ (data not shown). While the presence of the fluorescent dye did not significantly interfere with the normal interactions between nucleic acid chains in the example we have presented, this may not always be the case. We therefore recommend that on an individual basis the presence of a fluorescent dye does not interfere with biological activity.

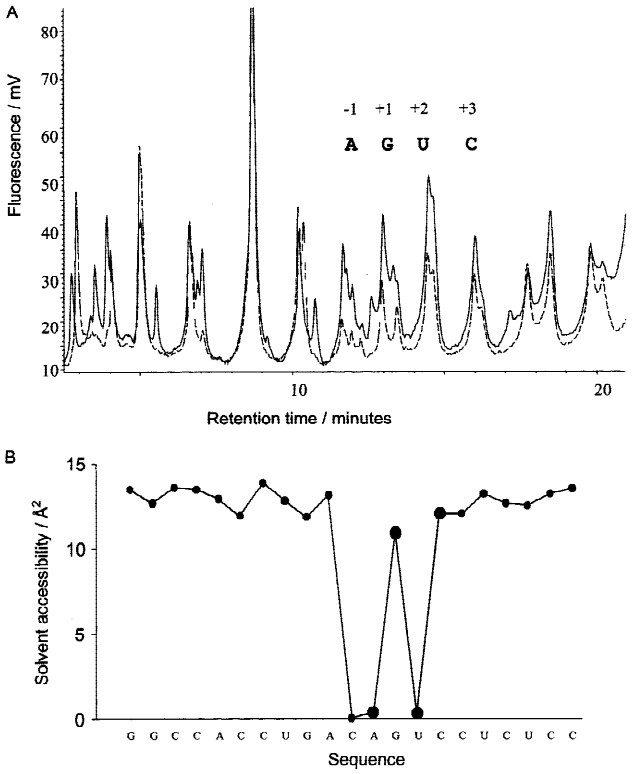

FIGURE 3. Hydroxyl radical footprint of the substrate in the docked ribozyme complex. **A:** Hydroxyl radical footprint of the fluorescently labeled substrate strand in the presence (dashed line) and absence (fixed line) of $Co(NH_3)_6^{3+}$. **B:** Chart of the solvent accessibility of the C4′ positions in the crystal structure of the hairpin ribozyme–inhibitor complex plotted against the sequence of the substrate strand. Hydroxyl radical footprinting data was overlaid onto the computed solvent accessibility, and protected regions are highlighted. The solvent accessibility was determined using NACCESS (Hubbard & Thornton, 1993).

CONCLUSIONS

Ion pair reverse phase liquid chromatography has been used to analyze the solvent accessibility of the substrate strand in the assembled hairpin ribozyme complex. This was performed by assessing the hydroxyl radical cleavage products of the fluorescently labeled substrate strand in the hairpin ribozyme complex. The cleavage products were directly quantified using in line data analysis to rapidly compare chromatograms and identify sites of solvent accessibility. Using this novel approach, both intra- and intermolecular RNA interactions can be analyzed in a convenient, quantitative, high-throughput manner.

MATERIALS AND METHODS

Synthesis and purification of oligonucleotides

Oligonucleotide synthesis was performed on an Applied Biosystems 394 DNA synthesizer using cyanoethyl phosphor-

FIGURE 4. Model illustrating the hairpin ribozyme–inhibitor complex from the crystal structure. The substrate strand is highlighted in black and the C4′ positions at which protection was observed in the hydroxyl radical footprinting experiments are shown as black spheres.

amidite chemistry. The fluorescent phosphoramidites were obtained from Glen Research. Oligonucleotides were deprotected using standard procedures (Young et al., 1999) and purified using denaturing PAGE and desalted using a Pharmacia NAP 10 column according to the manufacturer's instructions. Further purification was performed using IP RP HPLC using DNAsep column technology on the Transgenomic WAVE® system (Transgenomic, San Jose).

HPLC analysis

All samples were analyzed by IP RP HPLC on the WAVE Nucleic Acid Fragment Analysis System using a DNAsep column (Transgenomic, San Jose). The stationary phase of the column consists of a nonporous, alkylated poly(styrene-divinylbenzene) matrix. Analysis was performed using denaturing conditions at 75 °C. Chromatograms were analyzed under fluorescent detection at the appropriate excitation and emission wavelengths for the relevant fluorophore.

Base-catalyzed hydrolysis of RNA

One to five picomoles of fluorescently labeled RNA were incubated with 0.1 M $NaHCO_3$ (pH 8.5) in a total volume of 20 μL for 20 min at 95 °C, followed by the addition of 2 μL 0.1 M HCl overnight at 4 °C. Five microliters were then directly injected onto the DNAsep column and run using the following conditions for the fluorescently labeled substrate strand: buffer A 0.0025 M tetrabutylammonium bromide (Fisher HPLC), 0.1% acetonitrile, 1 mM (Na_4) EDTA, buffer B 0.0025 M, tetrabutylammonium bromide, 70% acetonitrile, 1 mM (Na_4) EDTA starting at 25% buffer B. The gradient was extended to 42% buffer B over 10 min, followed by an extension to 50% buffer B over 15 min at a flow rate of 0.9 mL/min. Fluorescently labeled loop B RNA, buffer A 0.0025 M tetrabutylammonium bromide (Fisher HPLC), 0.1% acetonitrile, 1 mM (Na_4) EDTA, buffer B 0.0025 M, tetrabutylammonium bromide, 70% acetonitrile, 1 mM (Na_4) EDTA starting at 35% buffer B. The gradient was extended to 50% buffer B over 15 min, followed by an extension to 56% buffer B over 15 min at a flow rate of 0.9 mL/min.

Hydroxyl radical footprinting

Hydroxyl radical footprinting was performed as described (Hampel et al., 1998) with minor modifications. Twenty picomoles of 5′ fluorescently labeled substrate strand was added to 100 pmol of the unlabeled loop A RNA and 100 pmol of the unlabeled 5′-loop B RNA. The mixtures were heated to 37 °C for 20 min both in the presence and absence and of 0.1 mM $Co(NH_3)_6^{3+}$. This procedure was repeated for the fluorescently labeled substrate strand in the absence of the loop A and B RNA. A solution of 20 mM iron (II) was freshly prepared using ferrous ammonium sulphate (Aldrich), and a complex was formed by mixing an equal volume with 40 mM EDTA (Sigma). Ten microliters of a 20 mM solution of sodium ascorbate (Sigma) and 5 μL of 0.6% H_2O_2 (Aldrich) were used in the footprinting reaction. After incubation at room temperature for 4 min, the reaction was stopped by the addition of 0.1 M thiourea in 0.2 M EDTA. The samples were analyzed on the Transgenomic WAVE system. One-fifth volume of the RNA was injected directly onto the column, equilibrated at 75 °C using the following conditions. Buffer A 0.0025 M tetrabutylammonium bromide (Fisher HPLC), 0.1% acetonitrile, 1 mM (Na_4) EDTA, buffer B 0.0025M, tetrabutylammonium bromide, 70% acetonitrile, 1 mM (Na_4) EDTA starting at 25% buffer B. The gradient was extended to 42% buffer B over 10 min, followed by an extension to 50% buffer B over 15 min at a flow rate of 0.9 mL/min.

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