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Short communication

Use of high-performance liquid chromatographic fractionation of large RNA molecules in the assay of group I intron ribozyme activity

D.E. Georgopoulos, M.J. Leibowitz*

University of Medicine and Dentistry of New Jersey–Robert Wood Johnson Medical School and Cancer Institute of New Jersey, 675 Hoes Lane, Piscataway, NJ 08854-5635, USA

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Abstract

Ion-pair reversed-phase high-performance liquid chromatography (HPLC), which has previously been used to fractionate double-stranded DNA molecules, can be applied to single-stranded RNA molecules in the size range of 200–1000 nucleotides. This procedure permits RNA molecules to be separated and recovered rapidly in liquid medium, thereby facilitating recovery. We have used this system to separate an in vitro transcription product containing a group I intron ribozyme from the intermediates and products of the splicing reaction, permitting rapid assay of ribozyme activity without the use of radioactivity.

Keywords: RNA; Ribozymes

1. Introduction

Self-splicing introns, including the group I and group II ribozymes [1,2], catalyze their own excision from precursor RNA, with ligation of the flanking exons. Specifically, in the splicing reaction of group I intron ribozymes, precursor RNA (denoted E1–I–E2, where E1 indicates the 5′-exon, I indicates the intron, and E2 indicates the 3′-exon) reacts with a guanosine nucleotide (G) to yield the E1 and GI–E2 intermediates. These further react to result in the ligated exons (E1–E2) and the excised guanylylated intron (GI) reaction products. Self-splicing reactions are typically assayed, using internally labeled radioactive precursor RNA, by polyacrylamide gel electrophoresis, followed by detection of radioactivity, to monitor the conversion of precursor RNA into the intermediates and products of the splicing reaction.

The reaction can also be assayed, by using non-radioactive precursor RNA, and following the incorporation of [α-^{32}P]guanosine nucleotide into the GI–E2 intermediate and excised GI product by gel electrophoresis. Alternatively, this reaction can be monitored by incorporation of radioactivity from the nucleotide into trichloroacetic acid (TCA)-insoluble material, although this rapid and convenient assay method, as previously reported, suffers from problems in quantitative reproducibility and high blanks [3]. A chromatographic method to separate RNA

*Corresponding author.

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molecules based on size has the potential of being much faster than gel electrophoresis. A preparative liquid phase system would also eliminate the losses incurred by gel purification and recovery of the RNA from a solid gel matrix.

Anion-exchange high-performance liquid chromatography (HPLC) has been used to separate small RNA molecules and purify them for crystallography and other physical studies [4]. This method has been applied to the assay of hairpin and hammerhead ribozyme-catalyzed RNA cleavage [5,6]. However, it has only been used for ribozymes and RNA substrates (for *trans*-acting ribozymes) of less than 50 nucleotides in length, but not for larger ribozymes, such as the self-splicing introns. Reversed-phase chromatography on non-porous alkylated poly(styrene–divinylbenzene) particles has been shown to resolve DNA oligonucleotides of less than 60 nucleotides in length [7]. Ion-pair reversed-phase HPLC on these same beads extended the range of sizes of DNA molecules resolved to make this method useful for restriction fragments or polymerase chain reaction (PCR) products [8,9], with resolution documented for fragments up to 1000 base pairs in length. Similar resolution of DNA fragments has also been attained using anion-exchange HPLC [10].

This paper describes the application of ion-pair reversed-phase HPLC to RNA molecules comparable in size to the DNA molecules separable by this method. This method makes possible a rapid analytical and preparative method for size fractionation of RNA molecules, which we have applied to the assay of the self-splicing reaction catalyzed by the Pc1.LSU group I intron ribozyme from *Pneumocystis carinii* sp. f. *carinii* [11].

2. Experimental

2.1. RNA samples

RNA standards included a 0.24–9.5 kilobase pair (kb) RNA ladder, a 0.16–1.77 kb RNA ladder (both from GIBCO-BRL, Rockville, MD, USA) and a 0.3–1.6 kb RNA ladder (Boehringer-Mannheim, Indianapolis, IN, USA). Preparation of the 660-nucleotide RNA molecule derived from the rRNA gene of *P. carinii* sp. f. *carinii* containing the Pc1.LSU group I intron was performed as described [12]. The DNA containing this intron and flanking exon fragments was amplified by PCR from a cloned DNA template, using DNA primers which added the seventeen nucleotide bacteriophage SP6 promoter [13]. The 660-nucleotide precursor RNA was transcribed from the amplified DNA using bacteriophage SP6 RNA polymerase (Promega, Madison, WI, USA), and the crude transcript RNA was separated from other reaction components by QIAquick Nucleotide Clean-Up spin column (Qiagen, Valencia, CA, USA). Splicing of crude precursor RNA was performed in the presence of 50 m*M* Tris–HCl (pH 7.5), 5 m*M* magnesium chloride, 0.4 m*M* spermidine and 10 μ*M* GTP for 30 min at 50°C. The reaction was stopped by addition of EDTA (tetrasodium salt, BioChemica MicroSelect Grade, Fluka, Milwaukee, WI, USA) to a final concentration of 2.5 m*M* and chilling on ice. In the splicing reaction, the precursor RNA (denoted E1–I–E2) is spliced in a two-step process to yield the splicing intermediates, E1+GI–E2, and final products, GI+E1–E2.

2.2. HPLC analysis

HPLC was performed using a Model HP1090M HPLC System (Hewlett-Packard Instruments, Wilmington, DE, USA) which had been modified to eliminate most metallic components coming in contact with the mobile phase, as recommended by the manufacturer of the column. Chromatography was performed on a DNASep column (Sarasep, San Jose, CA, USA), a 50×4.6 mm deactivated stainless steel column with titanium frits, with a stationary phase consisting of C_{18} alkylated non-porous poly(styrene–divinylbenzene) copolymer, 2.2 μm particle size [8]. Samples of 5 μl (for RNA standards) or 90 μl (ribozyme crude precursors or reaction products) were injected. RNA separation was performed as for DNA [8] with the following modifications. Eluent A was 0.1 *M* triethylammonium acetate (TEAA), pH 7.0, containing 0.1 m*M* EDTA; eluent B was eluent A containing 25% acetonitrile, prepared as described [9]. All eluent reagents used were HPLC grade from Fluka. HPLC-grade acetonitrile was from Fisher (Springfield, NJ, USA). Chromatography was performed at 55°C at a flow-rate of 0.75 ml/min, with

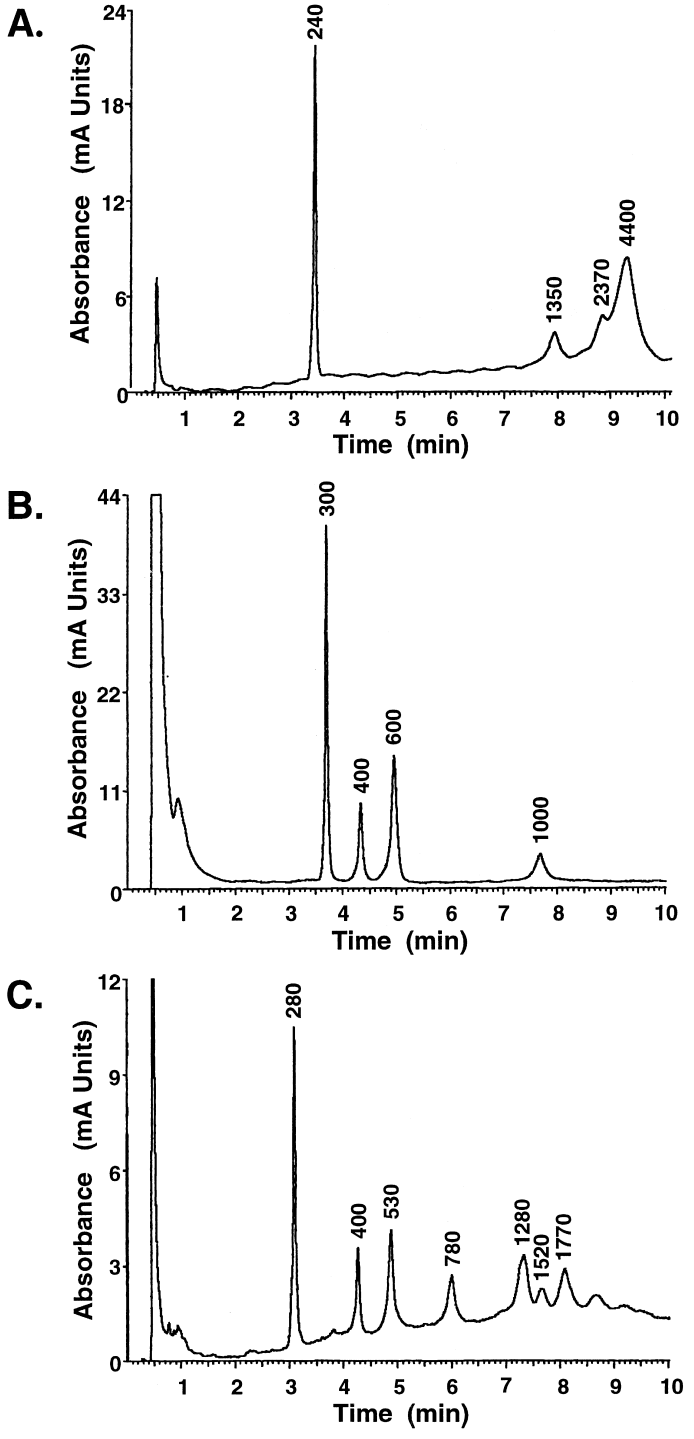

Fig. 1. Chromatographic resolution of RNA standards. Three sets of single-stranded RNA molecular mass markers were separated by HPLC on a DNASep column using the elution gradient profile described in the Experimental section. (A) 0.24–9.5 kb RNA ladder (GLBCO-BRL), (B) 0.3–1.6 kb RNA molecular mass marker III (Boehringer-Mannheim), (C) 0.16–1.77 kb RNA ladder (GLBCO-BRL).

the elution performed with the linear gradient profiles: 35 to 45% B (0 to 2 min), 45 to 55% B (2 to 12 min), 55% B (12 to 14 min), 55 to 100% B (14 to 15 min) followed by a 5 min wash in 100% B, and re-equilibration by a gradient from 100% to 35% B over 4 min.

3. Results and discussion

3.1. Fractionation of RNA molecules by HPLC

Fig. 1 shows the chromatographic resolution of various single-stranded RNA standards by HPLC. It can be seen that in the size range of 240 to 1000 nucleotides, different RNA species are well-resolved. Elution order is according to RNA chain length in the size range from 400 to 1000 nucleotides. As shown in Fig. 2, retention time can be used to estimate RNA size within this size range with accuracy comparable to that achieved by gel electrophoresis. It should be noted that chromatographic mobility of single-stranded RNA molecules may be determined by other factors in addition to size, as indicated by the apparently aberrant retention times of some of the RNA molecules with sizes outside the apparent linear portion of the plot in Fig. 2. Electrophoretic gel mobility of native single-stranded RNA molecules can also be affected by conformation and/or sequence (for example, [14]). Separation of RNA molecules in this size range suggested that this HPLC method could also be used to assay group I intron splicing.

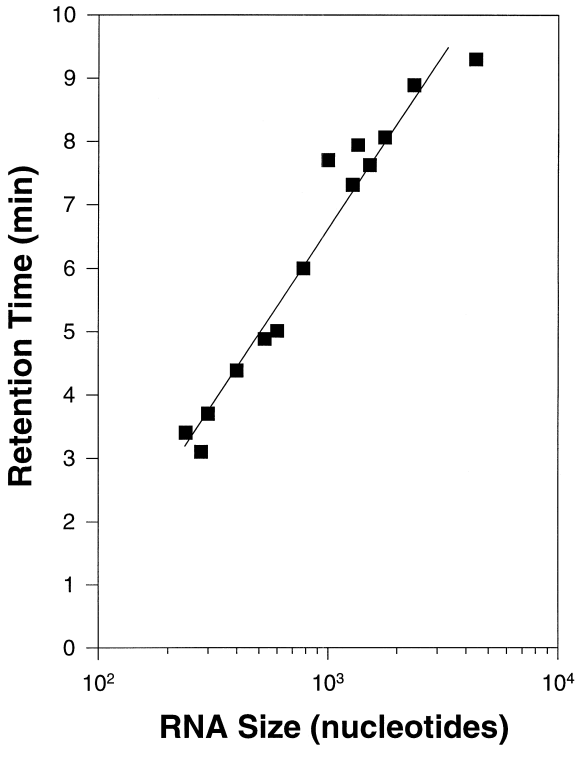

Fig. 2. Molecular mass vs. retention time. Molecular masses of separated single-stranded RNA markers shown in Fig. 1 were plotted vs. elution time.

3.2. HPLC assay of ribozyme activity

When the 660-nucleotide precursor RNA containing the Pc1.LSU group I intron is produced by in vitro transcription of a PCR product with this sequence downstream from a bacteriophage SP6 promoter, the crude transcription reaction contains the precursor RNA (E1–I–E2) along with the intermediates and products of the splicing reaction, which result from splicing occurring under transcription conditions [12]. For gel electrophoretic analysis of the splicing of in vitro radioactive transcript RNA, the precursor RNA is generally purified by preparative gel electrophoresis, to produce electrophoretically pure precursor RNA, which serves as the enzyme and substrate for the self-splicing reaction [12]. The use of radioactive RNA for ribozyme assays by gel analysis requires two electrophoresis steps: one to purify the precursor and another to assay the conversion of precursor to reaction intermediates and products. Not only is this time consuming, but internally labeled radioactive precursor tends to show significant radiochemical breakdown over the course of several days, limiting the usefulness of each preparation. For this reason, we assayed ribozyme activity of a crude non-radioactive precursor using the HPLC method described here.

As can be seen in Fig. 3A, the crude transcription reaction contains the predicted mix of RNA species, including the 660-nucleotide precursor RNA and the other species in descending size order: GI–E2 (382 nucleotides), GI (355 nucleotides), E1–E2 (305

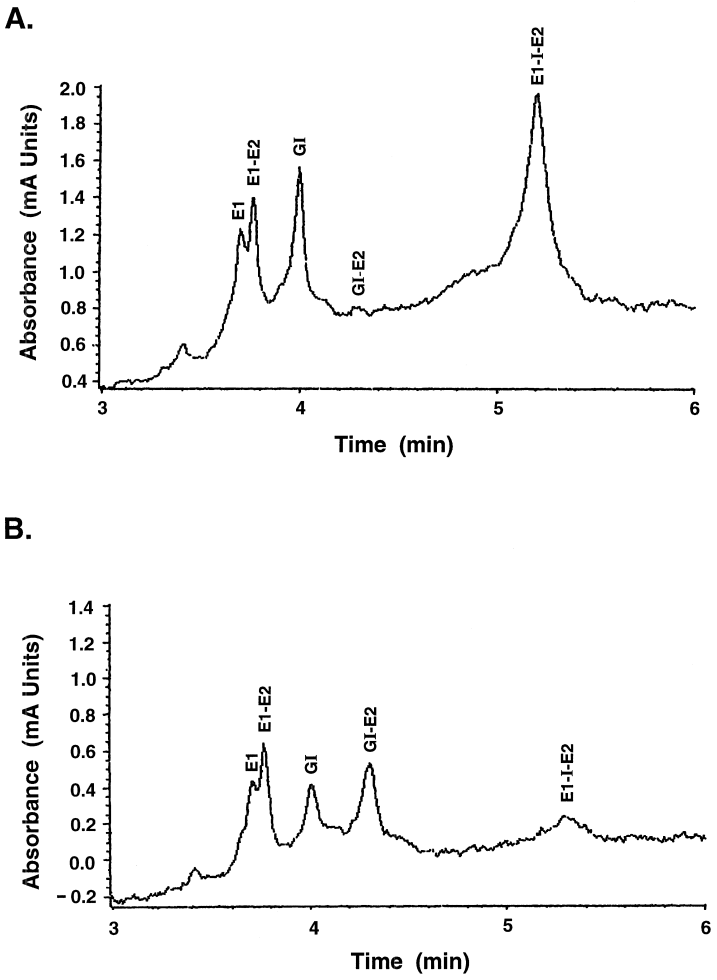

Fig. 3. HPLC chromatographic analysis of splicing of 660-nucleotide precursor RNA containing Pc1.LSU group I intron. (A) Unreacted 660-nucleotide RNA transcript chromatogram, indicating positions of precursor (E1–I–E2), intermediate (GI–E2 and E1), and final product (GI and E1E2) peaks. (B) Reacted RNA transcript chromatogram showing progress of group I splicing reaction, indicating disappearance of E1–I–E2 precursor peak and increase of GI–E2 intermediate.

nucleotides) and E1 (279 nucleotides) [12]. Peak collection and analysis by agarose gel electrophoresis confirmed the identification of the splicing product species (data not shown). In this chromatogram, the level of GI–E2 is barely detectable, while the precursor is a major peak. When the same sample was analyzed after allowing the splicing reaction to occur (Fig. 3B), the precursor peak was markedly reduced while the GI–E2 intermediate became a major species. Thus, HPLC can be used instead of gel electrophoresis to fractionate RNA species in the group I intron ribozyme assay, which can now be performed without the use of radioactivity.

The HPLC method described here should also be applicable to the analysis and purification of other RNA molecules of up to 1 kb in length.

4. Conclusion

The present method allows HPLC to be used for

the rapid fractionation of RNA molecules of up to 1 kb in length.

Acknowledgements

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