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Short Communication

Enrichment of biologically active U1 small nuclear RNAs by ion-exchange high-performance liquid chromatography

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ABSTRACT

The use of ion-exchange high-performance liquid chromatography in conjunction with preparative electrophoresis to facilitate the purification of biologically active snRNAs is described. Separation of total nuclear RNA from a *Bombyx mori* cell line was done with a Bio-Rad MA7 plasmid column in a HRLC 500 system. Individual fractions were subjected to electrophoresis through 14% polyacrylamide gels for identification. High levels of U1 RNA were confirmed by Northern analysis with a human U1 probe. Biological activity of RNAs from the column was demonstrated by their ability to incorporate ^{32}P-AMP at the 3′ end. Ion-exchange chromatography provides a rapid, automated method for purifying large amounts of RNAs that can then be utilized in further studies.

INTRODUCTION

This paper describes a method for separating small nuclear RNAs (snRNAs) by ion-exchange high-performance liquid chromatography (HPLC) in conjunction with preparative electrophoresis and aims at providing a rapid automated method for purifying specific snRNA species. The snRNAs are relatively short (*ca.* 80–300 nucleotides), non-ribosomal, non-transfer RNAs. These uracil-rich molecules (thus the name U series snRNAs) exist associated with at least seven proteins as small nuclear ribonucleoprotein particles (snRNPs). Each snRNP is composed of one molecule or U1, U2, or U5 snRNA [1]. U4 and U6 exist together in the same snRNA [2]. Peptides B, B′, D, D′, E, F and G are common to all U snRNPs. Some U snRNPs contain specific proteins, for example, the proteins 70K, A and C are found only associated with U1s. The snRNP complexes have been shown to participate in crucial cellular processes which include splicing of pre-mRNA precursors by U1, U2, U4, U5 and U6 [3–6]; 3′ editing of most histone mRNA precursors by U7 [7]; cleavage/polyadenylation of mRNA by U11 [8]; and ribosome biogenesis by U3, U8 and U13 [9].

Of particular interest are the recent preparative electrophoretic isolations of U1

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snRNA which revealed the existence of U1 isoforms; this was established in *Xenopus laevis* by Forbes *et al.* [10], in mouse by Kato and Harada [11], in chicken by Roop *et al.* [12] and in the silkmoth *Bombyx mori* by Adams and coworkers [13,14]. In addition, experimental evidence shows that U1 isoforms are tissue-specific and developmentally expressed [10, 13–16]. This may be indicative of their possible involvement in the regulation of gene expression at the RNA splicing level. Since most biochemical studies that could be performed to study structure–function relationships of these U1 variants require nano-and microgram quantities of individual isoforms, it is impractical to purify these RNAs by just preparative acrylamide electrophoresis. It is therefore advantageous to use HPLC for the enrichment of U1 isoforms in order to provide an efficient method to start purifying them and further study their involvement in pre-mRNA splicing *in vivo* and *in vitro*.

EXPERIMENTAL

Instrumentation

A Bio-Rad HRLC 500 series system consisting of two series 1350 soft start pumps, a Model 7125 injector, an HRLC system interface, and a 100-μl fixed-volume titanium steel loop was used. The column was a Bio-Rad MA7, 50 × 7.8 mm plasmid column, and detection was accomplished using a Bio-Rad monitor set at 260 nm. The Acer 900 personal computer system and Bio-Rad HRLC software were interfaced for data collection and analysis [17].

Mobile phase

Eluent A consisted of 5 *M* urea and 20 m*M* $NaPO_4$, pH 6.7 (283.5 ml of 40 m*M* NaH_2PO_3, 216.5 ml of 40 m*M* Na_2HPO_3/l). Eluent B consisted of eluent A with 1.5 *M* KCl, pH 6.7. The eluents were autoclaved, filtered through a 0.22-μm membrane and degassed by vacuum.

Sample preparation

Bm-N, a *Bombyx mori* ovarian-derived permanent cell line, was grown and subcultured in TC-100 medium as previously described [18]. Total nuclear RNA was extracted [19]. Nuclear RNA samples were dissolved in 99 μl distilled water and 1 μl 1 *M* Tris–HCl, pH 8.0. Each sample was injected into the loop at 23°C using a 1-ml syringe. After collecting the fractions, the samples were precipitated twice in three volumes of 100.0% ethanol at room temperature followed by centrifugation at 12 000 × *g* for 15 min to remove the KCl and urea. Prior to acrylamide gel analysis the samples were dissolved in 1 × TBE loading buffer (0.089 *M* Tris, 0.089 *M* boric acid, 0.05 *M* EDTA and trace amounts of the bromophenol blue and xylene cyanol electrophoretic marker dyes in 50% formamide, 20% glycerol, pH 8.3), heated at 65°C for 4 min, immediately chilled in ice water and loaded onto acrylamide gels for analysis.

Gel electrophoresis

The presence of snRNAs was established for each fraction by running each one through 14% polyacrylamide gels (8 *M* urea; 1 × TBE, 0.089 *M* Tris, 0.089 *M* boric acid, 0.05 *M* EDTA and trace amounts of bromophenol blue and xylene cyanol, pH 8.3). Electrophoresis was typically 2.5 h at 100 V constant power, until the xylene

cyanol marker migrated to the bottom of the gels. Gels were stained with ethidium bromide (10 μg/μl in water) for 5 min, destained twice in distilled water for 15 min and viewed under UV light. The gels were subsequently photographed.

Electrotransfer of nuclear RNA

The filters were cut to size, soaked in 1 × TBE and placed on the gels. Gels and filters were then placed between two pieces of Whatman No. 1 paper and set up on a Hoefer Model TE52 electroblotting apparatus with the filter side facing the positive electrode. The apparatus was placed in ice water and allowed to run for 2 h. The buffer was kept in circulation using a magnetic stir bar. The filter was then removed and baked at 80°C for 2 h under vacuum to fix the nucleic acid onto 0.1-μm Nytran membrane (Schleicher & Schuell).

Nucleic acid hybridization

The probe, a human U1 clone in the pSP6-64 [20] plasmid was radioactively labeled by random priming [21] using ^{32}P α-deoxy ATP. The filters were prehybridized in hybridization buffer [50% formamide, 10% Denhart's solution, 20% sodium dodecyl sulfate (SDS), 20 × SSC (3 *M* NaCl, 0.3 *M* sodium citrate · $2H_2O$, pH adjusted to 7.0 with 1 *M* HCl) and 13 μg/μl *E. coli* DNA] at 42°C overnight. The human U1 radioactive probe was denatured by boiling for 10 min, immediately placed in ice water and added to the hybridization buffer. This solution was then used for hybridization under the same conditions for 24 h. The filter was washed in a 1 × SSC, 0.1% SDS solution and exposed to Kodak XAR-5 film overnight at − 135°C with two intensifying screens.

Addition of poly(A) chains to the 3'-ends of snRNAs

The addition of poly(A) tracts was done as described by Devos *et al.* [22] utilizing 1.7 μg of peak 3 (Fig. 1) RNA per reaction and ^{32}P α-ATP. Autoradiography was done for two days at room temperature with Kodak XAR-5 film.

RESULTS AND DISCUSSION

The gradient profile of eluent B used for the separation and analysis of Bm-N

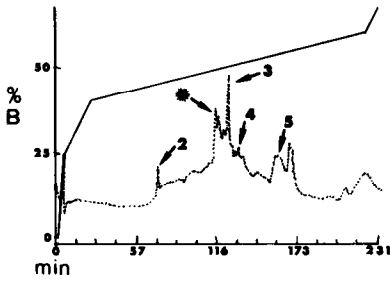

Fig. 1. Representative ion-exchange chromatography of total nuclear RNA from the Bm-N cell line. (Quantity of total nuclear RNA injected was 105 μg.) The abscissa represents the time in minutes. The *y*-axis is percent eluent B. Numbered arrows point to peaks collected. Their RNAs were loaded into wells 2, 3, 4 and 5 of Fig. 2, respectively. Peak No. 3 was eluted at 120 min at 50.4% eluent B. Peak marked with an asterisk contained mainly mature and precursor tRNA.

snRNAs was: 0–3 min, 100.0% A/0% B; 3–8 min, 100.0–75.0% A/0–25.0% B; 8–26 min, 75.0–59.8% A/25.0–40.2% B; 26–223 min, 59.8–40.0% A/40.2–60.0% B and 223–232 min, 40.0–32.8% A/60.0–67.2% B. A linear gradient that starts at a low salt concentration and ends at a high salt concentration was used. Although other gradient profiles were employed, the parameters represented above proved to be the method of choice for optimizing the sharpness and separation of the peaks.

Eluent peaks from nuclear extracts were identified by collecting each corresponding fraction and separating them by polyacrylamide gel electrophoresis. Peak 3 in Fig. 1, eluted from the column at a concentration of 50.4% eluent B (0.76 *M* KCl), was identified as a fraction enriched in U1 snRNA (Fig. 2, lane 3). Fig. 2 also shows the composition of some of the other eluent peaks after gel electrophoresis, and shows that peak 4 (lane 4) was enriched in U2 and U3 snRNAs. Peak 5 in Fig. 1 (lane 5) was mainly composed of high-molecular-weight RNA. The additional peaks to the right of peak 5 that were eluted at higher salt concentrations contained high-molecular-weight RNA that failed to penetrate the 14% polyacrylamide gel and remained at the origin (data not shown). Peak number 2 in Fig. 1 was composed of small-molecular-weight RNAs that migrated out of the gel into the reservoir buffer while the peak marked with an asterisk contained mainly mature and precursor tRNA (data not shown).

The high level of U1 snRNA in fraction 3 was confirmed by electrotransfer of the RNA in the gels onto 0.1 μm Nytran filters and hybridization with the human U1 clone. This is illustrated by Fig. 3, lane 3, which shows an autoradiogram displaying a strong signal in the area of U1 snRNA.

Former work in this area by Adams *et al.* [13], has shown the area of U1 snRNA to be between 5.8S and 5S RNAs, very close to 5.8S RNA. This area can be easily identified by using RNA markers during gel electrophoresis as shown in Fig. 2,

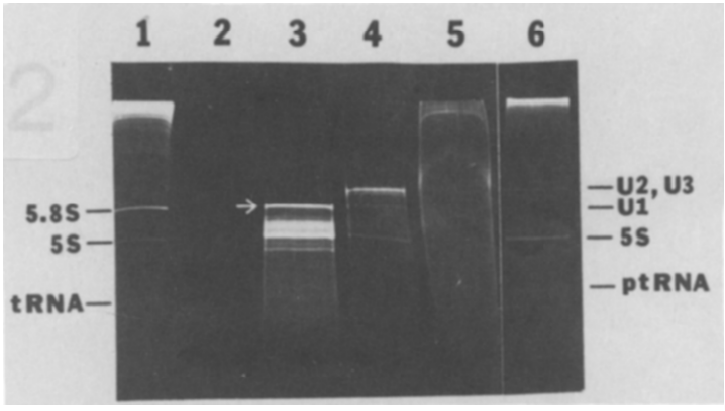

Fig. 2. Electrophoretic separation of fractions collected during the HPLC fractionation represented in Fig. 1. Well 1, total cytoplasmic RNA as a marker; mature tRNA is shown in this fraction. Well 6, total nuclear RNA (total nuclear RNA was run on a separate gel, but under the same conditions as the gel used for this experiment). Precursor tRNA (ptRNA) can be seen in this fraction. RNA in well 3 is enriched for U1, U4, 5S, U5 and U6 while well 4 contains mainly U2 and U3 snRNAs. White arrow points to U1 snRNA. RNAs were visualized by ethidium bromide staining.

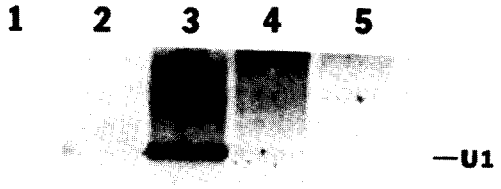

Fig. 3. Autoradiogram of gel in Fig. 2 after electroblotting and probing with a cloned human U1 gene. Notice the strong signal from the U1 area identified in Fig. 2, lane 3. The slow-moving smear in wells 3 and 4 represents high-molecular-weight DNA.

lanes 1 and 6. U1 snRNA was found to be about 0.5 mm below 5.8S RNA after electrophoresis as described in the Experimental section.

In order to determine the usefulness of this HPLC enrichment technique in some molecular biology studies, the biological activity of fraction 3 was tested by determining the snRNAs' capacity to incorporate, consecutively, 5'-adenosine ribonucleotide monophosphate (AMP) at its 3'-hydroxyl end by poly(A) polymerase (RNA adenyltransferase). Since snRNAs lack a poly(A) tail, it is essential to polyadenylate them before cDNA production and cloning. Fig. 4 shows the polyadenylation of this fraction. The snRNAs present in greater mass (*e.g.*, 5S) exhibit greater AMP

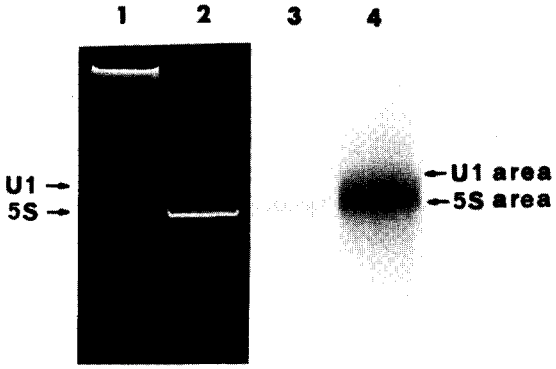

Fig. 4. Electrophoretic separation and autoradiography of fraction 3 (Fig. 2, lane 3) at different times during poly(A) tract addition. snRNAs in wells 1 and 2 were visualized by ethidium bromide staining while wells 3 and 4 represent an autoradiograph of the ^{32}P-labelled RNA. Well 1, total nuclear RNA as a marker; wells 2–4, fraction 3 at reaction incubation times of 0, 5 and 20 min, respectively. Wells 1 and 2 were run on a separate gel, but under the same conditions as the autoradiographed gel used in this experiment.

incorporation. Notice that there was an overall increase in radioactive signal as incubation time augmented from 5 to 20 min. As expected, due to the different sizes of poly(A) tracts incorporated into individual snRNAs [poly(A) polymerase ceases polyadenylation irregularly subsequent to initiation], the bands and radioacitivy became more diffuse with reaction time.

This study demonstrates some potential for ion-exchange HPLC in conjunction with electrophoresis to facilitate the purification of specific species of biologically active snRNAs. These purified biologically active snRNAs, in turn, can be utilized in experiments probing their structure and function.

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