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PURIFICATION OF POLY(A)-MESSENGER RIBONUCLEIC ACID BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Polyadenylated messenger ribonucleic acid [poly(A)-mRNA] was purified by reversed-phase high-performance liquid chromatography (RP-HPLC). Partially purified poly(A)-mRNA was prepared from rat liver polysomes by magnesium precipitation followed by affinity chromatography on an oligo(dT) cellulose column. Translatable poly(A)-mRNA, as assayed by *in vitro* translation in a rabbit reticulocyte system, was resolved from non-translatable RNA by RP-HPLC. The separation appears to be due to the presence of poly(A) residues on the 3′ terminus of the mRNA.

INTRODUCTION

Recently, high-performance liquid chromatography (HPLC) on silica packings has been used to resolve messenger RNA (mRNA) based on the size of the RNA[1,2]. To achieve the separation, either reversed-phase HPLC (RP-HPLC) with an ion-pairing agent[1] or gel filtration HPLC[2] were used. Although these studies demonstrate the usefulness of HPLC for mRNA, there was a limit to the resolution of individual mRNAs in a complex mixture.

Over 90% of the eukaryotic mRNAs (with the exception of histone mRNAs) that enter the cytoplasm contain a 3′-polyadenylate segment [poly(A)] of *ca.* 200 residues[3]. This feature of eukaryotic mRNAs has been used to enrich poly(A)-containing mRNAs by affinity chromatography on poly(U)-Sepharose[4] or oligo(dT)-cellulose[5]. The present study demonstrates the resolution of poly(A) mRNA from RNA that lacks poly(A) by RP-HPLC.

MATERIALS AND METHODS

Rabbit globin mRNA was purchased from Bethesda Research Labs. (Gaithersburg, MD, U.S.A.), *E. coli* transfer RNA (tRNA) from Sigma (St. Louis, MO, U.S.A.) and [^{35}S]methionine (1200 Ci/mmol) from Amersham (Arlington Heights, IL, U.S.A.).

Polysomal RNA was isolated from the liver of a female Sprague-Dawley rat by magnesium precipitation according to the method of Palmiter[6]. The RNA was enriched for poly(A)-mRNA by two consecutive passes through an oligo(dT)-cellulose

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column[5] (Collaborative Research, Lexington, MA, U.S.A.) unless noted otherwise.

RP-HPLC was performed on a 150 × 4.6 mm I.D. stainless-steel column packed with Synchropak RP-P C_8 (octylsilyl, 6.5 μm particle size, 300 Å pore size) from Synchrom (Linden, IN, U.S.A.). The column was slurry-packed in the laboratory by a Shandon column-packing instrument (Jones Chromatography, Columbus, OH, U.S.A.). The chromatography was performed with a Beckman 324 gradient chromatograph equipped with two Model 100A pumps, Model 421 controller and Model 210 injector (Beckman, Berkeley, CA, U.S.A.). All gradient chromatography was done at 37°C unless otherwise noted.

The RNA samples dissolved in water were treated at 65°C for 4 min immediately prior to injection into the chromatograph. Upon injection of the sample, the gradient was started and consisted of a linear gradient of acetonitrile in 0.01 *M* sodium sulfate (pH 6.0) unless otherwise specified. The eluted RNA was detected by UV-absorbance at 260 nm with a LDC Spectromonitor III variable-wavelength detector (Laboratory Data Control, Riviera Beach, FL, U.S.A.). To RNA fractions, purified by RP-HPLC, 25 μg tRNA were added, and after ethanol precipitation and desalting on Sephadex G-50, they were treated with 1 m*M* sulfuric acid and precipitated with ethanol. The purified RNA fractions required these steps before subsequent RP-HPLC or *in vitro* translation.

In vitro translation was performed in a rabbit reticulocyte lysate system described in ref. 7, obtained as a kit (Bethesda Research Labs.). Each reaction mixture (40 μl) contained 5 μCi of [^{35}S]methionine. The translation reaction was assayed for incorporation of labeled methionine into protein by two methods: In the first method, an aliquot of the reaction was decolorized and the tRNA hydrolyzed in sodium hydroxide and hydrogen peroxide, and the labeled protein was precipitated with trichloroacetic acid in the presence of 50 μg of bovine serum albumin (BSA) onto filters. The dried filters were placed in scintillation fluid[8] and the radioactivity was determined in a liquid scintillation counter. The second method of analysis of the labeled translation products employed hydrophobic interaction HPLC, following sodium hydroxide hydrolysis of the tRNA in an aliquot of the reaction. To the treated aliquot, 50 μg of BSA and 5 ml of 0.1% trifluoroacetic acid (TFA) were added. This was then fractionated with a HPLC "mini-column" system[9] by pumping the sample onto a column of RP-P C_8 packing using a Milton Roy Mini Pump (Laboratory Data Control). The column was then washed with 5 ml of 0.1% TFA and then with 5 ml of 5% 1-propanol in TFA. The translation products were eluted with 50% 1-propanol in 0.1% TFA and then lyophilyzed. The samples were dissolved in the primary solvent and analyzed by the gradient chromatograph at 37°C in 0.1% TFA at a flow-rate of 1 ml/min and a linear gradient of 5–50% 1-propanol (0.5%/min) started 2.5 min after injection[10]. Fractions of 1 ml were collected, scintillation fluid containing 33% Triton X-100 was added, and the radioactivity was determined in a liquid scintillation counter.

RESULTS

Rat liver polysomal RNA, enriched with respect to poly(A)-RNA by two successive passes through an oligo(dT)-cellulose column, was analyzed by RP-HPLC. Using an octyl-bonded silica packing with a solvent system of 0.01 *M* sodium sulfate

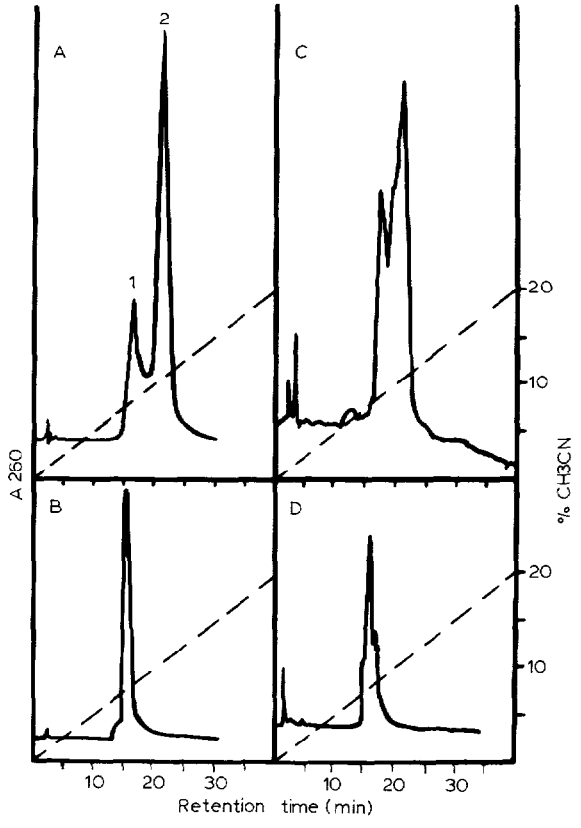

Fig. 1. RP-HPLC of RNA. The separations were performed at a flow-rate of 1 ml/min with a linear gradient of acetonitrile (CH_3CN) in 0.01 *M* Na_2SO_4 (pH 6.0). The dashed line indicates the acetonitrile gradient (0.5%/min) and the solid line the UV-absorption at 260 nm. (A) Oligo(dT)-enriched rat liver RNA, eluted as peak 1 and peak 2; (B) rat liver RNA not retained by oligo(dT); (C) rabbit globin mRNA; (D) *E. coli* tRNA. Attenuation: A, C, D is 0.01 a.u.f.s.; B is 0.5 a.u.f.s.

(pH 6.0) and a linear gradient of acetonitrile (0.5%/min), this poly(A)-enriched RNA was separated into two distinct peaks (Fig. 1A). Rat liver RNA that was not retained by the oligo(dT)-cellulose column eluted with the same retention time by RP-HPLC as the first peak of the poly(A)-enriched RNA (Fig. 1B). When rat liver polysomal RNA is enriched for poly(A)-RNA by a single pass through an oligo(dT)-cellulose column and then analyzed by RP-HPLC, peak 1 increased in height and peak 2 was unchanged from that observed in Fig. 1A (data not shown). This RP-HPLC fractionation of rat liver RNA, purified by affinity chromatography on oligo(dT)-cellulose, apparently separated the poly(A)-mRNA from the RNA lacking poly(A) in this polysomal preparation. This separation is further supported by RP-HPLC analysis of rabbit globin mRNA, which is polyadenylated, and *E. coli* tRNA, which lacks poly(A). The globin mRNA was eluted as a complex of peaks with about the same retention time (Fig. 1C) as peak 2 of the rat liver RNA, whereas tRNA was eluted with the same retention time (Fig. 1D) as peak 1 of the rat liver RNA. Therefore,

TABLE I

EFFECTS OF SOLVENT SYSTEM ON RP-HPLC OF RAT LIVER RNA

The apparent selectivity (α_{app}) and resolution (R_s) were determined for peak 1 and peak 2 after RP-HPLC analysis of oligo(dT)-enriched rat liver RNA. The selectivity is expressed as α_{app} because α is defined by isocratic elution systems. The chromatography conditions are described in Fig. 1. (A) Various salts in aqueous solution were tested, keeping concentration (0.01 *M*), pH (6.0), and acetonitrile gradient constant. (B) Various organic solvents were tested, keeping the 0.01 *M* sodium sulfate (pH 6.0) constant.

	α_{app}	R_s
A. Aqueous solutions		
Sodium sulfate	1.30	1.06
Sodium phosphate*	1.48	0.93
Sodium carbonate	1.07	0.35
Sodium chloride	1.15	0.50
Potassium sulfate	1.39	0.68
Potassium phosphate	1.19	0.60
Potassium acetate	1.32	0.51
Lithium sulfate	1.52	0.69
Magnesium sulfate	No recovery	
Zinc sulfate	No recovery	
Ammonium sulfate	No recovery	
Water	No recovery	
B. Organic solvents		
Tetrahydrofuran	1.13	0.50
1-Propanol	1.21	0.65
Acetonitrile	1.30	1.06
Ethanol	1.32	0.83
Methanol	1.37	0.91

* Recovery with sodium phosphate as aqueous component was only 30% of that with sodium sulfate.

RNA having a retention time corresponding to peak 2 is apparently poly(A)-containing mRNA whereas the RNA in peak 1 lacks poly(A).

Using oligo(dT)-purified rat liver polysomal RNA, variations of the aqueous and organic components of the mobile phase were studied for effects on the relative separation of peak 1 from peak 2 by RP-HPLC. The results were expressed in terms of the separation parameters of apparent selectivity (α_{app}) and resolution (R_s)[11]. The selectivity is expressed as α_{app} because α is defined by isocratic elution systems. As shown in Table I, changing salt in the aqueous solution did not alter the selectivity significantly for the two peaks. However, the highest R_s value (widest separation and narrowest band-width) was observed with sodium sulfate. The absolute recovery of polysomal RNA with the sodium sulfate system was 93%, as determined by UV absorbance at 260 nm of the total RNA before and after RP-HPLC. Although sodium phosphate had a similar R_s value as sodium sulfate, the recovery with sodium phosphate was 30% of that with sodium sulfate. Moreover, with some solutions (magnesium sulfate, zinc sulfate and ammonium sulfate, and water alone) either peak 1 or peak 2 could not be recovered. The concentration of 0.01 *M* sodium sulfate was the minimum required for optimal resolution and recovery of these peaks of RNA. In the presence of sodium sulfate, the α_{app} values for organic solvents of various pola-

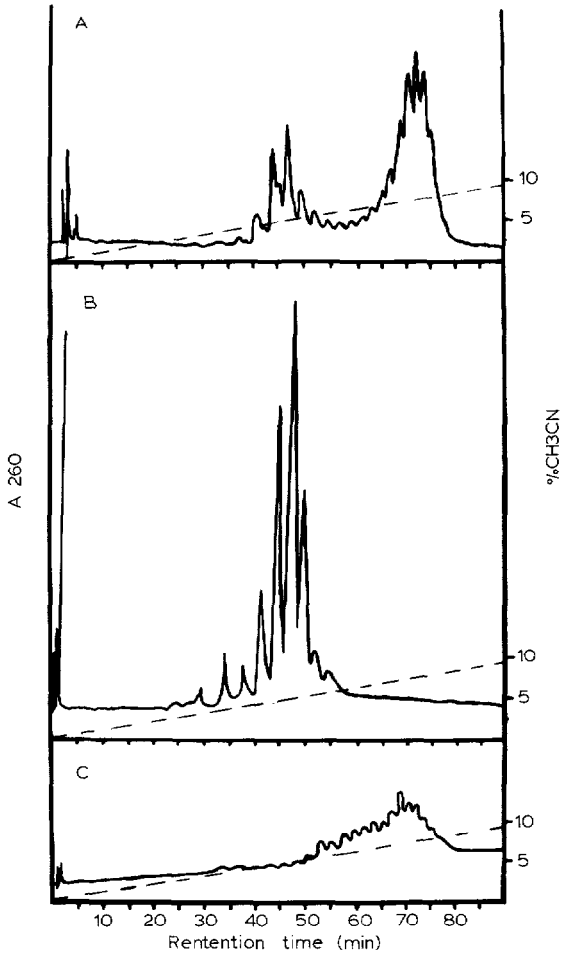

Fig. 2. RP-HPLC of rat liver RNA and rabbit globin mRNA. The separations were performed as in Fig. 1 except that the flow-rate was 2 ml/min and the acetonitrile gradient (dashed line) was 0.1%/min. (A) Oligo(dT)-enriched rat liver RNA; (B) rat liver RNA not retained by oligo(dT); (C) rabbit globin mRNA. Attenuation: A is 0.01 a.u.f.s.; B is 0.2 a.u.f.s. and C is 0.02 a.u.f.s.

rities were similar. However, the R_s values for the least polar solvents, tetrahydrofuran and 1-propanol, were significantly lower than for acetonitrile. Methanol and ethanol which are more polar than acetonitrile gave slightly lower R_s values than acetonitrile. The effect of pH on the separation of rat liver RNA into peak 1 and peak 2 was examined with an acetonitrile gradient in sodium phosphate because of the buffering capacity of this salt over the pH range of interest. The R_s values did not differ significantly between pH 5.0–7.6. However, below pH 5.0 both resolution and recovery were poor (data not shown). Finally, the effect of temperature on the resolution of peaks 1 and 2 was examined with the sodium sulfate–acetonitrile solvent system. Temperatures below 37°C lengthened the retention time and broadened the peaks, but chromatograms at 45°C and 37°C showed no difference (data not shown).

In an attempt to resolve the poly(A)-RNA peak 2 of rat liver further, a shallow gradient of acetonitrile (0.1 %/min) in sodium sulfate was tried. When oligo(dT)-purified rat RNA (Fig. 2A) was compared with rat RNA that is not retained by oligo(dT) (Fig. 2B) after chromatography with this gradient, peak 2 of rat liver RNA apparently had been separated into several components. However, when globin mRNA, which is 80% pure, was analyzed by this gradient, it also had several components with the same retention time as peak 2 of the rat liver RNA (Fig. 2C). Therefore, the shallow acetonitrile gradient apparently revealed the heterogenity of the poly(A) lengths of the globin mRNA and the rat liver peak 2[12].

In vitro translation in the rabbit reticulocyte system of the rat liver polysomal RNA and the rabbit globin mRNA before and after RP-HPLC confirms that the RNA in peak 2 is mRNA (Table II). The results show that the various portions of the globin mRNA peak complex fractionated by RP-HPLC (see Fig. 1C) retained translational activity after RP-HPLC. Similarly, the HPLC peak 2 of the rat liver RNA directed protein synthesis in the *in vitro* translation assay. However, peak 1 of this RNA preparation did not increase the incorporation of labeled methionine into protein above that in the blank, containing no added RNA. The labeled protein products from the *in vitro* translation of the RP-HPLC-purified globin mRNA and rat liver peak 2 RNA were analyzed by a system of protein separation by hydrophobic interaction HPLC (Fig. 3). The labeled products translated by rat liver peak 2 RNA had a broader distribution than those of the globin mRNA, thus demonstrating the greater complexity of the rat liver peak 2 mRNA. In addition, the labeled protein profile was identical for each fraction of the globin-mRNA peak complex in Fig. 1C, showing that the mRNA content of each fraction was identical (data not shown).

TABLE II

IN VITRO TRANSLATION OF RP-HPLC PURIFIED RNA

In vitro translation with rabbit reticulocyte lysate was performed with and without additions of RNAs before and after purification of the RNA by RP-HPLC. Incorporation of [^{35}S]methionine into protein was determined in an aliquot of the reaction by trichloroacetic acid precipitation (TCA ppt.). The following RNAs were added: globin mRNA (0.5 μg) before HPLC; HPLC globin mRNA-A, -B and -C, corresponding to the fractions collected after RP-HPLC of 1.0 μg globin mRNA eluted between 16.5–18.2 min, 18.2–21.5 min and 21.5–23.5 min, respectively, in Fig. 1C; oligo(dT)-purified rat liver RNA (0.5 μg) separated by HPLC into peak 1 and peak 2, corresponding to the fractions collected after RP-HPLC of 1.2 μg RNA eluted between 15–16.6 min and 19.3–22.7 min, respectively, in Fig. 1A.

Addition	*Radioactive TCA ppt. (cpm)*
Blank	360
Globin mRNA	9684
Globin mRNA-A	840
Globin mRNA-B	1272
Globin mRNA-C	2049
Liver RNA	2270
Liver RNA, peak 1	341
Liver RNA, peak 2	1549

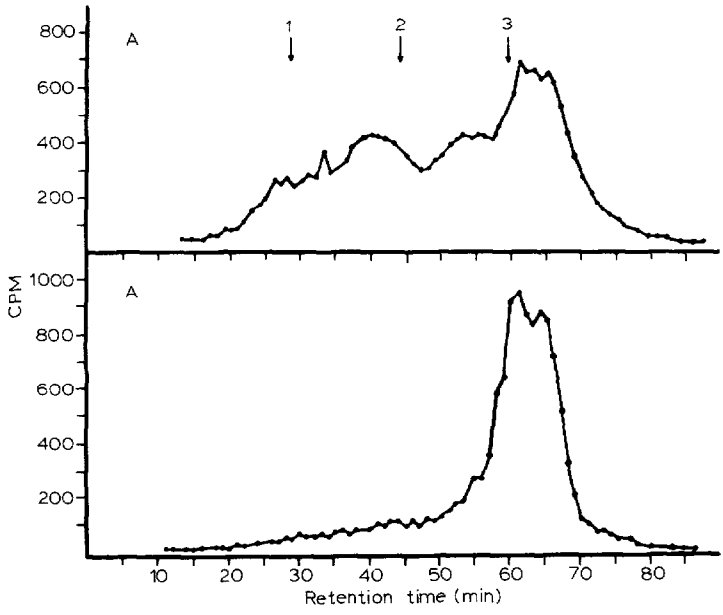

Fig. 3. Hydrophobic interaction HPLC of translation products after minicolumn fractionation. An aliquot of the translation reaction mixture from rat liver peak 2 (A) and globin mRNA peak C (B), as described in Table II, where eluted at a flow-rate of 1 ml/min from a RP-PC8 column with a linear gradient (0.5%/min) of 1-propanol in 0.1% TFA. Fractions (1 ml) were collected, and radioactivity was determined by liquid scintillation counting. Elution of standard proteins: 1 = $ACTH_{1-24}$; 2 = $ACTH_{1-39}$; 3 = BSA.

DISCUSSION

Using RP-HPLC, poly(A)-containing RNA has been separated from RNA lacking poly(A). The resolution of these classes of RNA requires a mobile phase composed of sodium sulfate with a linear gradient of acetonitrile. It is presumed that the reversed-phase chromatography of RNA requires ion-pairing by a cation as suggested by Nguyen *et al.*[1]. However, the requirements for the resolution of poly(A)-RNA appear to be rather strict with regard to the aqueous component of the mobile phase. The present study demonstrates the separation of RNA by a chemical property of the solute rather than by size as has been previously reported for RNA fractionated by ion-pairing HPLC[1] and gel filtration HPLC[2]. This is supported by the fact that mRNA sizes range from 400 to 4000 nucleotides[13], rabbit globin mRNA being at the lower end of this range[14], yet bulk liver poly(A)-mRNA and globin mRNA are not separated by the present RP-HPLC system. We do not know the minimum length of poly(A) required for elution of mRNA in the poly(A) peak nor whether the separation is specific for poly(A) residues.

Using the RP-HPLC method described here, highly purified poly(A)-mRNA can be isolated from an enriched poly(A)-RNA preparation within 30 min in quantitative yield. This RP-HPLC-purified mRNA retains biological activity as measured by *in vitro* translation.

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