Resolution of Ribonucleic Acids by Sepharose 4B Column Chromatography†

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ABSTRACT: Ribonucleic acids were resolved by molecular sieve chromatography on columns of Sepharose 4B. The elution positions of messenger ribonucleic acids were determined by detection of polyadenosine tracts and by support of protein synthesis in a messenger-dependent cell-free system. The elution position of other ribonucleic acid species from the Sepharose 4B was determined by formamide–sucrose density gradient centrifugation. Resolution of ribonucleic acids by this column was not dependent on molecular weight but rather on other properties such as secondary structure or the presence of poly(adenylic acid). The elution profiles of ribonucleic acids on cross-linked Sepharose 4B differed markedly from those on conventional Sepharose and appeared to depend on molecular size alone. There was diminished resolution of high molecular weight ribonucleic acids on such columns.

The purification of mRNAs to homogeneity is an important problem in molecular biology. The ability to synthesize cDNAs[1] and complementary strands for molecular cloning purposes is dependent on sources of pure mRNA template, free of unknown and, therefore, potential biologically hazardous contaminants. Toward this end, we have addressed ourselves specifically to the purification of high molecular weight mRNAs on a preparative scale.

There exist multiple techniques to separate RNA species from a mixture of total RNA samples. A method which has been introduced recently is Sepharose 4B molecular sieve chromatography, first described by Novaković and Petrović (1972) and Petrović et al. (1973, 1974a–c) and currently being applied to separation of mRNAs from total oviduct RNAs (Woo et al., 1974). The separation is due not only to molecular weight, as might be anticipated, but also to secondary structure which apparently plays an important role in determining chromatographic behavior of different RNA species.

The use of Sepharose 4B, as described by Woo et al. (1974), presents the advantage of being a preparative method, in which large amounts of RNA can be processed, particularly as the first in a series of sequential purification steps. The behavior of total RNAs from different tissues on Sepharose 4B appears to follow the same basic pattern using the criterion of optical absorbance, making this technique generally suitable to the resolution of RNAs from a variety of sources. However, the elution position of mRNA populations from different tissues seems to vary. The mRNA distribution profiles reported by Woo et al. (1974) are all similar, with the exception of mRNA from lactating mammary gland. Additional variations have been observed in the present studies. In this communication we report the chromatographic profiles of RNAs extracted from several connective tissue sources, including rat calvaria. The mRNA populations had elution profiles entirely different from those reported by Woo et al. (1974).

Our studies suggest that the elution position of mRNA populations on Sepharose 4B molecular sieve chromatography can vary and must be established for each tissue.

In addition, we report here the chromatographic results obtained with a new cross-linked Sepharose 4B, in which the RNA profiles differed from those of conventional Sepharose 4B.

Materials and Methods

Materials. Sepharose 4B and Sepharose CL-4B were purchased from Pharmacia Fine Chemicals AB. Fresh commercial wheat germ was supplied by General Mills, Inc., Vallejo, Calif. Tritiated proline was purchased from New England Nuclear and [^{3}H]poly(U) from Schwarz/Mann. All other chemicals were purchased from Calbiochem. All solutions were prepared under sterile conditions and treated with 0.05% DEPC. Glassware, including all columns, was autoclaved.

RNA Extraction. Total RNA was extracted from newborn rat calvaria with phenol. Approximately 300 calvaria were incubated overnight in Eagle's minimum essential medium with 500 μCi of [^{3}H]uridine at 37 °C. After incubation, the tissue was washed several times with cold media and placed in 250 mL of the first extraction buffer: 50 mM NaAc; 1 mM EDTA; 1% sodium deoxycholate and 0.5% sarkosyl at pH 5.0. An equal volume of phenol–chloroform–isoamyl alcohol (125:125:2.5) was added and the mixture was shaken for 15 min at 37 °C. After centrifugation at 2000 rpm, the aqueous phase was removed and kept on ice. The phenol and the interface were extracted twice more with 125 mL of 50 mM Tris containing 1 mM EDTA at pH 9.0. After centrifugation, all aqueous phases were combined and extracted twice more with phenol. The RNA was precipitated by addition of 0.03 volume of 4 M NaCl and 2.1 volumes of ethanol and kept at 20 °C overnight. After centrifugation, the pellet of RNA was dried with nitrogen, dissolved in DEPC-treated water, and its con-

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[1] Abbreviations used: cDNA, copy DNA, the DNA prepared from an mRNA template using reverse transcriptase obtained from avian myeloblastosis virus; mRNA, messenger ribonucleic acid; RNA, ribonucleic acid; EDTA, ethylenediaminetetraacetic acid; DEPC, diethyl pyrocarbonate; hnRNA, heterogeneous nuclear RNA; rRNA, ribosomal RNA; Cl_3AcOH, trichloroacetic acid; MAK, methylated albumin–kieselguhr; MASA, methylated albumin–silicic acid; BD-cellulose, benzoylated DEAE-cellulose; DEAE, diethylaminoethyl; Sepharose CL-4B, the cross-linked form of Sepharose 4B; poly(U), polymer of poly(ribouridylic acid); GC content, the proportion of total nucleotides in an RNA sample which is guanylic acid and cytidylic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

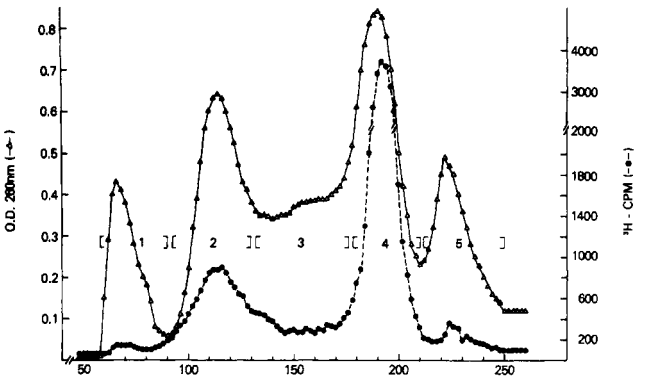

FIGURE 1: Sepharose 4B column chromatography of total RNA extracted from rat calvaria. Ribonucleic acid was extracted with phenol at room temperature as described under Materials and Methods. Fifteen milligrams of labeled RNA was applied to the column and chromatography was carried out as described. Absorption was recorded continuously at 260 nm and radioactivity determined directly. The indicated fractions were pooled and lyophilized.

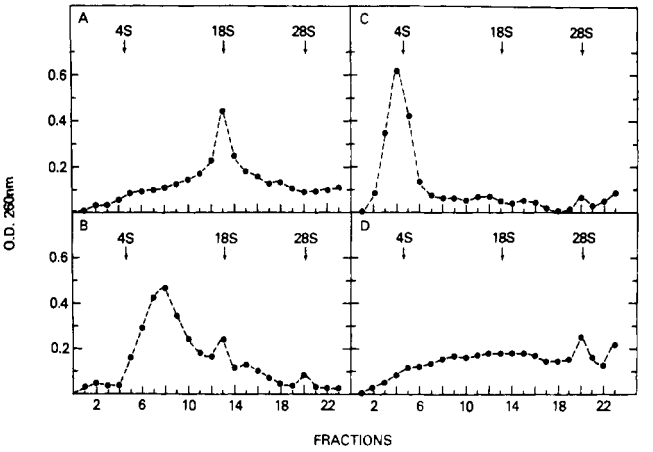

FIGURE 2: Formamide-sucrose gradient sedimentation centrifugation of pooled lyophilized fractions from Figure 1. The pooled fractions shown in Figure 1 were suspended in buffer and applied to the formamide-sucrose gradient tubes as described under Materials and Methods. Following centrifugation, fractions of 0.4 mL were collected with continuous recording of A_{260}. Radioactive contents of these fractions followed closely the profiles of A_{260}. (A) Pooled fractions of peak 2; (B) pooled fractions of peak 3; (C) pooled peak 4; and (D) pooled peak 5.

centration determined by absorption at 260 nm, assuming that 1 A_{260} = 50 μg of RNA.

Chromatography of Sepharoses 4B and CL-4B. A Sepharose 4B column (2.5 × 100 cm) was equilibrated with 0.1 M NaAc containing 1 mM EDTA at pH 5.0. The RNA sample was dissolved in the same buffer at a concentration of 3 mg/mL, and 15 mg was applied to the column. Downward chromatography was performed at 4 °C. Fractions of 5.5 mL were collected at a flow rate of 10 mL/h with continuous monitoring of absorbance at 260 nm. Radioactivity was determined in 0.2-mL aliquots after addition of 10 mL of scintillation liquid. The fractions of RNA were pooled, lyophilized, dissolved in water, and precipitated with ethanol. Chromatography on Sepharose CL-4B was identical with that on Sepharose 4B.

Poly(A) Determination. The presence of poly(A) sequences was assayed by hybridization with [^{3}H]poly(U) by the method of Gillespie et al. (1972). A standard 50-μL hybridization mixture contained: 3 × SSC buffer (0.45 M NaCl and 45 mM sodium citrate); 10 mM Tris, pH 7.2; 50% formamide; 0.02 μCi of [^{3}H]poly(U) (1.0 nmol with a specific activity of 18 mCi/mmol); 5 μg of RNA. The samples were incubated for 24 h at 36 °C and cooled to 30 °C and 1 mL of a solution containing 10 mM Tris (pH 7.2), 10 mM $MgCl_2$, 0.5 M NaCl, 5 μg/mL of boiled pancreatic ribonuclease, and 20 μg/mL of deoxyribonuclease I was added. After 2 h incubation, samples were chilled, an equal volume of 10% Cl_3AcOH was added, and the specimens were kept on ice for 30 min. After filtration on glass fiber filters, the radioactivity was determined by addition of 5 mL of toluene scintillation mixture and counted on a Beckman-LS 335 liquid scintillation system.

Sucrose Gradient Centrifugation. The RNA fractions were characterized by sucrose gradient centrifugation in the presence of formamide by modification of the method of Anderson et al. (1974). Gradients from 5–20% sucrose in a buffer containing 0.1 M LiCl, 50 mM EDTA, 0.2% sodium dodecyl sulfate, 10 mM Tris (pH 7.4), and 50% formamide were used. The RNA was heat denatured at 60 °C for 10 min before being applied to the gradient. Centrifugation was carried out at 25 °C for 15 h at 32 000 rpm in the L2 Beckman/Spinco centrifuge using an SW-41 rotor. Fractions of 0.4 mL were collected and the radioactivity was determined by addition of 0.5 mL of water and 10 mL of Insta-Gel and counted.

Wheat Germ Cell-Free System. Wheat germ S_{23} was prepared as described by Shih and Kaesberg (1973). The reaction mixture was prepared as described by Harwood et al. (1975). A 50-μL standard reaction mixture contained: 3.2 mM NaAc, 0.1 M KAc, 2 mM dithiothreitol, 20 mM Hepes (pH 7.2), 1 mM ATP, 20 μM GTP, 8 mM creatine phosphate; 2.5 μg/L creatin phosphokinase; 25 μM of 19 amino acids but not proline; 5 μL of [^{3}H]proline (specific activity, 1.37 × 10^4 cpm/pmol), 20 μL of wheat germ S_{23}, and 1–3 μg of RNA. The samples were incubated for 90 min at room temperature, 8 μg of pancreatic ribonuclease was added, and the incubation was continued for 30 min at 37 °C. The samples were chilled, and 50 μL of 10% Cl_3AcOH containing 0.5% tannic acid with 2 mM unlabeled proline was added. The Cl_3AcOH precipitates were filtered onto fiber glass filter, washed with ethanol, and dried, and radioactivity was determined by the addition of 5 mL of toluene scintillation mixture and counted.

Results

Fifteen milligrams of total RNA extracted from rat calvaria was chromatographed on Sepharose 4B. The elution profile shown in Figure 1 was obtained. The RNA was separated into four well-resolved peaks, with a shoulder between the second and third peak (fraction no. 3). The profile of radioactivity followed the pattern of absorbance at 260 nm, although proportionality was not consistent throughout. The RNA was pooled in five fractions as indicated in Figure 1 and, after concentration by ethanol precipitation, the RNA in each fraction was further characterized.

Figure 2 demonstrates the profile obtained on sucrose density gradient centrifugation of an aliquot of each pooled fraction. In Figure 2, profile A represents the second pooled peak from Sepharose 4B, which contained one major component with a sedimentation coefficient of 18 S. Figure 2B represents the third pooled fraction from Sepharose 4B, which contained several RNA species with different sedimentation coefficients. Four main peaks were observed with a major fraction sedimenting as a broad peak at approximately 9 S. Figure 2C corresponds to the fourth pooled fraction and contained primarily one RNA species sedimenting at 4S, although some other species of higher sedimentation value were present at lower concentrations. Figure 2D represents the last pooled

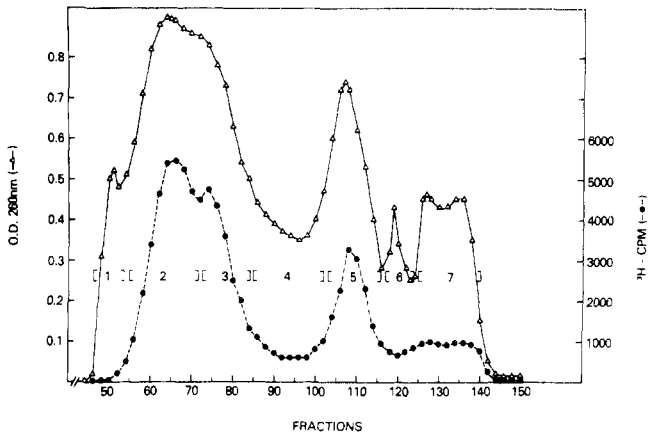

FIGURE 3: Cross-linked Sepharose 4B column chromatography of total RNA extracted from rat calvaria. This was carried out as in Figure 1.

TABLE I: Characterization of mRNA Content of Isolated RNA Fractions from Sepharose 4B.[a]

RNA Fraction No.	Poly(U) Hybridization (cpm/μg of RNA)	[^{3}H]Proline Incorp (cpm/μg of RNA)
1	46	80
2	63	100
3	147	1370
4	136	2000
5	0	0

[a] The pooled peak fractions indicated from the Sepharose 4B column in Figure 1 were lyophilized, dissolved in water, precipitated with ethanol, and kept at −20 °C overnight. After centrifugation, the pellet was dried with N_2 and dissolved in water, and the concentration of RNA was determined by A_{260}. Aliquots containing the required concentration of RNA, as described in Materials and Methods, were measured for mRNA content both by [^{3}H]poly(U) hybridization and by the ability to support protein synthesis in a cell-free wheat germ system.

fraction to be eluted from the Sepharose column, containing a well defined peak at 28 S, plus a heterogeneous population of RNAs present throughout the gradient. The first peak eluting from the column sedimented as a pellet on the gradients (not shown) contained less than 1% of the total radioactivity. It was resistant to alkaline hydrolysis and, therefore, was identified as DNA.

From these data, the peaks eluting from the Sepharose column were identified in order as: first DNA, followed by 18S rRNA, a major peak of 4S tRNA, with 28S rRNA the last peak to be eluted. The shoulder between 18S and 4S RNA, corresponding to pooled fraction 3, contained several species of RNA, with a range of sedimentation coefficients.

The elution position of mRNAs on the Sepharose 4B column was determined by two separate measures: the presence of poly(A) sequences, and the ability to support translational activity in a cell-free protein-synthesizing system. These were determined for each pooled fraction. The results in Table I demonstrate good correlation between the two assays, poly(A) content and the activity in a cell-free protein-synthesizing system, and indicate that the mRNA population was distributed between the third and fourth peaks. This elution position of mRNA on Sepharose 4B was corroborated when hemoglobin mRNA was chromatographed and eluted in a single narrow peak immediately following 18S rRNA (figure not shown). Since total RNA extracted from rat calvaria contains a heterogeneous population of mRNAs, it would be anticipated that the mRNAs elute in a broad peak compared with the narrow profile obtained with a homogenous mRNA population.

The major drawback of RNA chromatography on Sepharose 4B was the prolonged time required to carry out the procedure (48 h). Although chromatography was carried out at 4 °C, and, under conditions as free as possible of ribonuclease contamination, some degradation can occur during such procedures. For these reasons, a new product, the cross-linked Sepharose 4B, was examined. This column resin had several advantages over the conventional Sepharose 4B, such as increased flow rate, so that time required for the chromatography could be shortened (24 h), increased stability, permitting washing of the resin with extreme conditions of alkaline or acid pH to remove traces of residual RNAs and ribonucleases, and ability to be autoclaved, so that RNA can be chromatographed under sterile and scrupulously ribonuclease-free conditions. When the same amount of rat calvaria RNA was chromatographed under identical conditions as on conventional Sepharose 4B, a different profile was obtained. The first and second peaks were poorly separated. The second peak was not homogeneous and contained two peaks of radioactively labeled material plus a double peak at the end of the chromatographic profile which was not detected on conventional Sepharose 4B. The RNA from the elution profile was pooled into seven peaks, as shown in Figure 3, and each was characterized by sucrose density gradient sedimentation. Fraction 1, as in the case of the conventional Sepharose 4B, was identified as DNA. Fraction 2 from Sepharose CL-4B contained primarily 28S RNA with some contamination by 18S RNA. Fraction 3 corresponded to 18S RNA with a small component of 28S, as expected, since resolution of these fractions, as shown in Figure 3, was poor. Fraction 4 contained a heterogeneous mixture of RNAs varying in size with some residual 18S and 28S RNA. Fraction 5 contained a single peak with a sedimentation coefficient of 4 S. Fractions 6 and 7 contained a single peak each, both with a sedimentation coefficient of less than 4 S. No difference could be detected between fractions 6 and 7 by the sucrose density gradient centrifugations. This technique is known not to resolve species with small differences in molecular weight, such as 4S tRNA and 5S rRNA.

The presence of mRNAs was identified again by poly(A) content and by activity in a messenger-dependent cell-free protein-synthesizing system. The results are shown in Table II and, once again, many of the mRNA species were present in pooled fractions 3 and 4, although fraction 6 contained some activity also.

The enrichment in mRNA after chromatography on each of the two Sepharose resins, as measured by translational activity in the cell-free protein-synthesizing system, is shown in Table III. The mRNA fractions following chromatography on Sepharose 4B were enriched by a factor of 8.4, compared with the starting material. The cross-linked Sepharose 4B gave poor enrichment (×1.4), which was anticipated since resolution of high molecular weight RNAs in this column was poor.

Discussion

Although chromatography on agarose beads has been widely used to separate high molecular weight macromolecules such as microsomes (Tangen et al., 1973), polysomes (Darnbrough et al., 1973; Eschenfeldt and Patterson 1975), proteins (Killander et al., 1964), and viruses (Fridborg et al., 1965), the

TABLE II: Characterization of mRNA Content of Isolated RNA Fractions from Sepharose CL-4B.[a]

RNA Fraction No.	[^{3}H]Poly(U) Hybridization (cpm/μg of RNA)	[^{3}H]Proline Incorp (cpm/μg of RNA)
1	0	0
2	59	0
3	125	184
4	384	372
5	90	82
6	168	141
7	50	45

[a] The pooled peak fractions from the cross-linked Sepharose 4B column indicated in Figure 4 were measured as in Table I.

TABLE III: Enrichment in mRNAs Following Chromatography on Sepharose 4B Columns.

RNA Sample	Act. in a Cell-Free Protein Synthesizing System (cpm/μg of RNA)	Enrich-ment
Crude	400	
Sepharose 4B, peak 3 + 4	3370	8.4
Sepharose CL-4B, peak 4	556	1.4

resolution of nucleic acid is a more recent application. Resolution on these columns heretofore has been observed to be dependent on molecular weight. However, this is not the case for nucleic acids, as observed in this and in an earlier publication (Petrović et al., 1973, 1974a,c; Woo et al., 1974). The unusual chromatographic behavior of RNA may be explained by the major role of secondary structure, as suggested by Petrović et al. (1973, 1974a-c). High ionic strength, which stabilizes secondary structure, increases retention of RNAs on Sepharose. The ionic strength at which RNAs are eluted from the column depends on the GC content and the molecular weight of the RNA and tends to decrease as molecular weight increases (Petrović et al., 1973). This property of the RNA and Sepharose 4B to interact provides a useful method to separate RNAs with small differences in molecular weight but which differ in base composition. Such RNA species would be difficult to separate by other means. An additional major advantage offered by this technique is the high capacity of the column resin, in that large amounts of RNA can be chromatographed without compromising resolution. Variations of ionic strength and porosity of the Sepharose result in good separation of rRNAs from different species (Petrović et al., 1973, 1974b). Separation of 28S from 18S rRNAs (Petrović et al., 1974a), DNA and double stranded RNA (Petrović et al., 1974c), mRNA (Holmes and Bonner, 1973), and mRNA from other RNAs (Woo et al., 1974; Rosen et al., 1975) can be obtained. Rosen et al. (1975) have been able to separate the ovalbumin mRNA from DNA, tRNA, and much of the ribosomal RNA using Sepharose 4B. Total separation of the specific ovalbumin mRNA from other mRNAs was not completely achieved since the mRNAs eluted together as a class, in a position preceding 18S rRNA. This indicates that common features of mRNAs such as the poly(A) fragment present in most may endow them with particular chromatographic properties on Sepharose.

The results presented in this communication are similar to the observations reported by Rosen et al. (1975). The order of elution, DNA, 18S, 4S, and 28S RNA, was the same. The 28S rRNA peak was retained on the column, though on the basis of size alone this large RNA might be expected to be excluded from the molecular sieve and be the first RNA to be eluted. The major difference observed between our results and those obtained by Rosen et al. (1975) was the elution position of the mRNA fractions. The mRNA populations as reported by Rosen, with one exception, elute just prior to, and together with 18S rRNA. On the other hand, mRNA population in our results eluted consistently in a broad peak following the 18S elution position. Rosen observes that with the single exception of the mRNA from lactating mammary gland, which elutes in two peaks, one preceding and one following the 18S peak, mRNA from all other tissues elute prior to the 18S peak. The RNA used in this communication was obtained from connective tissues.

Specific mRNAs, such as collagen mRNA, were present in the fraction containing the bulk of mRNAs. This was determined by translation of sucrose gradient fractions in the cell free system. The product obtained with the 28S rRNA fraction was characterized as collagen by digestion with collagenase and immunoprecipitation with specific antibodies (Zeichner et al., 1976).

Total RNA extracted from other connective tissue sources, such as chick embryo calvaria, chick embryo wings and legs, or a rat chondrosarcoma, had similar profiles, with the mRNAs eluting after the 18S peak. Not only RNA from connective tissues but also hemoglobin mRNA eluted after the 18S peak. These results might be explained by slight variations in conditions, such as the initial pH and salt concentrations of the sample when applied to the column. Such an effect has been observed with MAK (Mandell and Hershey, 1960), MASA (Stern and Littauer, 1968), and BD-cellulose (Sedat et al., 1967) column chromatography. Ribonucleic acids have more random structures at low salt concentrations, but once applied to the column, the freedom of movement is restricted, and the salt concentration of the elution position is determined in part by the range of salt concentrations to which the RNA was exposed when first applied to the column. Variations in profiles, on the other hand, might also represent authentic differences between the structures of mRNAs from different tissues.

Another significant observation is the difference in resolution of RNAs between cross-linked Sepharose 4B and the conventional Sepharose 4B. It was anticipated that the two column techniques would resolve RNAs in a similar fashion. That they did not may be due to the fact that the charged groups involved in covalent cross-linking are no longer available for interaction with the polyanionic RNA polymers. Similarly the cross-linked Sepharose is 50% less efficient than the conventional Sepharose for affinity chromatography, because it has fewer charged groups available (Pharmacia Catologue, 1975). With the cross-linked Sepharose, the RNA species eluted strictly in accordance to molecular weight, 28S rRNA being the first to elute, followed by 18S rRNA, and 4S. Further separation of low molecular weight RNA species was also observed. The presence of covalent cross-links on the Sepharose alters the capacity for the resolution of RNAs. Resolution of high molecular weight RNAs was lost and low molecular weight RNAs became better separated. Thus, the resolution capacity of Sepharose 4B cross-linked resin tended to resemble that of the Sepharose with the next lower exclusion limit, Sepharose 2B. The aforementioned observations must be considered when gel filtration is to be used to separate RNAs. The Sepharose 4B and the Sepharose CL-4B resins each offer a different and distinct advantage, dependent upon the nature

of the resolution to be achieved.

Although it appears that separations of mRNAs by gel filtration does not differ from the results that can be achieved by poly(U)-Sepharose or oligo(dT)-cellulose, there are several advantages in each one of these methods. A problem with both poly(U)-Sepharose and oligo(dT)-cellulose is residual contamination with 28S rRNA. The use of Sepharose 4B chromatography as an initial step for mRNA purification can eliminate this problem. On the other hand, if the initial purification step is performed on poly(U)-Sepharose or oligo(dT)-cellulose, appropriate conditions of ionic strength on Sepharose 4B might give some degree of separation. The basis of this separation might be the GC content, size of the poly(A) fragment, secondary structure, proportion of the nonpoly(A)-bearing mRNAs, or nature of the nontranslated region.

Acknowledgments

The excellent technical assistance of Ms. Valoree Gift is gratefully acknowledged. We also thank Dr. Leslie J. Krueger for the gift of globin mRNA and for many useful discussions throughout the course of these experiments.

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