Journal of Chromatography, 564 (1991) 115–125
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5675

Separation of translationally active mRNAs by reversed-phase ion-pair high-performance liquid chromatography

C. A. VAN DER MAST*, D. HEKSTRA and H. O. VOORMA

Department of Molecular Cell Biology, University of Utrecht, Padualaan 8, 3584 CH Utrecht (The Netherlands)

(First received June 27th, 1990; revised manuscript received October 3rd, 1990)

ABSTRACT

An ion-pair high-performance liquid chromatographic method on C_4 columns was developed for the separation of mRNAs. The addition of methylmercuric hydroxide markedly influenced the separation according to length of these molecules. A method is given to recover minute amounts of translatable mRNA from the organic phase. The resolution of mRNAs improved with increasing pore size of the column support.

INTRODUCTION

The separation parameters of mRNAs are only the amount of negative charges, *i.e.* the length or molecular mass of these molecules, and the differences in hydrophobicity due to differing base compositions. As these nucleic acids also possess a secondary and a tertiary structure, the separation often is unpredictable and therefore totally empirical.

The problem is aggravated by the low percentage of mRNAs in relation to the other RNAs. Furthermore, the class of mRNAs constists of several thousand molecular species, in contrast to a lower defined number of the more abundant rRNAs and tRNAs.

Electrophoretic separation [1] offers a good resolution for the more abundant species, but the capacity is low and losses in recovery are high. The capacity problem can be alleviated by the use of ion exchangers. Anion exchangers are an obvious choice as they separate according to the charge of the molecule, and thus according to its length when the molecules are in a denatured state. Two forms of exchangers can be recognized, *i.e.* the normal exchangers and the mixed-mode ones. Examples of the first group are the TSK-DEAE.5PW, mono Q, Nucleogen-DMA and Nucleogen-DEAE columns [2–5]. Separation according to length has been effected up to a molecular mass of *ca.* 10^6 Da, but the resolution is rather low as the large molecules need supports with large pore sizes, which adversely affect the separation of the smaller molecules. The mixed-mode exchangers, such

0378-4347/91/$03.50

as Aminex A28 [6,7] and polyethyleneimine-coated silicas [8,9], also exhibit hydrophobic interactions and are suitable for the separation of small oligonucleotides and tRNAs.

Reversed-phase chromatography separates mRNAs according to differences in hydrophobicity, but offers no better than group separation at high capacities. Usually silica supports coated with C_8 or C_{18} are used [10,11].

Reversed-phase ion-pair (RPIP) chromatography has been used in the separation of nucleotides [12,13], oligonucleotides [14,15], restriction fragments [16] and RNAs [17]. As ion-pairing agents the positive alkylammonium ions have been successfully used. The separation is dependent on the amount of the ion-pairing compound bound to the molecule, which is a function of the length of this molecule. Elution is accomplished with a buffered gradient of organic solutions. The column material is usually of the C_{18} type. Parameters that influence the resolution are the hydrophobic properties of the constituent bases, the ion-pairing agent, the organic solvent employed in the gradient and the length of the RNA molecule. The ionic strength of the solvents, the pH, the temperature and the column properties are of lesser importance.

The non-ionic compound methylmercuric hydroxide not only acts as a powerful inhibitor of RNases but also effectively destroys the secondary structure of DNA and RNA at low concentrations by covalent attachment to, especially, the imino groups of thymidine, guanosine and uridine [18,19], which are essential for Watson–Crick base pairing. This disruption of the secondary structure should lead to increased binding of ion-pairing compounds and to elution according to the length of the nucleic acids from reversed-phase ion-pairing column material. The binding of methylmercuric hydroxide can be reversed by sulphydryl compounds [19].

The separated mRNAs can be isolated from the organic phase used in the elution of the columns by ethanol precipitation in the presence of carrier tRNA and glycogen.

EXPERIMENTAL

Chemicals

All chemicals were of Analar grade except acetonitrile, which was HPLC grade, and Agarose, which was of ultra-pure quality. Guanidinium-isothiocyanate and the unlabelled amino acids were purchased from Fluka (Buchs, Switzerland); 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), antifoam, ATP, GTP, sodium dodecyl sulphate, dithiothreitol, Agarose and oligo-dT-cellulose from Sigma (St. Louis, MO, U.S.A.); glycogen, creatine phosphate, creatine kinase and rat liver tRNA from Boehringer (Mannheim, Germany); caesium chloride, methylmercuric hydroxide and the ion-pairing agents from Janssen Chimica (Beerse, Belgium); and acetonitrile from Rathburn (Walkerburn, U.K.). All other chemicals were purchased from Merck (Darmstadt, Germany). [^{35}S]Methionine was from Amersham (Amersham, U.K.).

Sterilization

All glassware was sterilized overnight at 200°C. Most solvents were treated with 0.1% diethylpyrocarbonate and autoclaved. All plastic material was steam-sterilized or boiled in 0.5% sodium dodecyl sulphate (SDS), rinsed with 0.1 *M* NaOH and washed with sterile water.

Isolation of RNA

RNA was isolated by the guanidinium-isothiocyanate method [20] from livers of young rats that had fasted for 24 h. To every gram of liver were added 10 ml of a solution that consisted of 6 *M* guanidinium-isothiocyanate, 25 m*M* sodium-citrate (pH 7.0), 0.5% SDS, 0.1% antifoam A and 0.1 *M* β-mercaptoethanol. The solution was filtered through 0.45-μm Millipore filters (Millipore, Bedford, MA, U.S.A.).

The tissue was homogenized twice for 30 s in a Sorvall omnimixer. The homogenate was cleared at 20 000 *g* for 15 min. CsCl was added to the supernatant in a ratio of 1 g to 2.5 ml of fluid. The dense solution was layered onto 10 ml of CsCl solution in 70-ml polycarbonate bottles. The CsCl solution consisted of 5.7 *M* CsCl and 0.1 *M* EDTA (pH 7.0). The mixture was filtered as described above. After centrifugation at 140 000 *g* for 25 h at 20°C the supernatant was discarded and the RNA pellets were washed with 70% (v/v) ethanol and dissolved in 0.5% (w/v) SDS at 4°C. The solution was deproteinized by phenol extraction and RNA was precipitated with ethanol. Poly A-rich RNA was isolated with oligo-dT-cellulose, as previously described [21].

In some experiments globin mRNA from reticulocytes was used.

Separation of mRNAs

An LKB HPLC system equipped with a 2150 pump, a Rheodyne 7125 sample injector (100-μl loop), a 2158 UV detector with a 275-nm filter and a 2152 gradient controller was employed. The binary eluent consisted of two solutions: (A) 47% (v/v) acetonitrile (MeCN) in water, 5 m*M* methylmercuric hydroxide (CH_3HgOH), 5 m*M* tetrabutylammonium hydroxide (TBA), 5 m*M* H_3PO_4 or 2.5 m*M* H_2SO_4 (pH 7.1–7.6); (B) 70% (v/v) acetonitrile in water. The solutions were continuously deacrated with helium. The flow-rate was 0.5 ml/min, and the pressure 40–60 bar.

Two types of Nucleosil C_4 column (particle size 5 μm, pore diameter 30 nm; and particle size 7 μm, pore diameter 100 nm) were obtained from Macherey & Nagel (Düren, Germany). The column dimensions were 250 mm × 4 mm I.D. Chromatography was performed at room temperature, and fractions were collected manually.

Concentration of RNA after RPIP chromatography

The first attempts to isolate fractionated RNA by evaporation of the liquid phase led to extensive breakdown of these molecules. The RNA molecules could

be isolated only by ethanol precipitation after the addition of 2.5 μg of rat liver tRNA and 20 μg of glycogen, which act by coprecipitation. Precipitation was then achieved by the addition of ammonium acetate–acetic acid (pH 5.2) to a final concentration of 0.3 *M* and 2.5 volumes of cold 96% ethanol. After overnight incubation at −20°C the RNA was collected by centrifugation in an Eppendorf centrifuge at 8500 *g* during 1 h. The pellets were dried in vacuo, dissolved in 6 μl of sterile water and dialysed on floating membranes (Millipore VSWP, 0.025 μm, diameter 25 mm) in petri dishes against 50 m*M* potassium acetate (pH 7.0) and 1 m*M* CH_3HgOH (as an inhibitor of RNases) for 2–3 h in the cold. The drops were collected, and the corresponding sites on the membranes were washed with 1–2 μl of sterile water to counter loss of RNA and of liquid. This was added to the samples, which were then stored at −70°C. The end volume was between 5.6 and 7 μl.

Cell-free systems

The isolated mRNAs were assayed for translation in 10- or 12.5-μl systems, which contained 20 m*M* HEPES–KOH (pH 7.6), 1 m*M* ATP–$Tris_3$, 0.4 m*M* GTP–$Tris_3$, 2 m*M* dithiothreitol, 5 m*M* creatine phosphate–$Tris_2$, 0.2 U of creatine kinase (EC 2.7.3.2), 2 m*M* magnesium acetate, 120 m*M* potassium acetate, 0.1 m*M* spermine · HCl, 50 μ*M* each of nineteen unlabelled amino acids, 3 μCi of [^{35}S]methionine (specific activity 1200 Ci/m*M*), 0.75 μg of rat liver tRNA, 2 μg of pH 5 enzymes as a source of amino-acyl synthetases, and elongation factors from rat liver [22] and 0.075 and 0.25 A_{260} units of 40S and 60S rat liver ribosomal subunits, respectively. Crude initiation factors were precipitated with ammonium sulphate from a high salt wash of rabbit reticulocyte ribosomes according to Schreier and Staehelin [23]. Of fraction A (0–40% saturation of ammonium sulphate) 4 μg were added to the systems, and 10 μg of fraction BC (40–70% saturation) were used. The mRNA to be tested comprised 3 μl of the cell-free system. The incubations were performed at 37°C during 90 min. Samples of 2 μl were spotted on Whatman 3 MM filters, treated with hot 10% trichloroacetic acid, twice with cold acid and once with 96% ethanol, dried and counted in 2 ml of xylofluor (Lumac, Landgraaf, The Netherlands) in an LS 6000 SE liquid scintillator from Beckman (Fullerton, CA, U.S.A.). The counting efficiency was 70%.

Electrophoresis

Proteins were electrophoresed according to the method of Laemmli [24] on 15% polyacrylamide gels. Staining was performed either with Fast Green or Coomassie Brilliant Blue R. The gels were then treated with Enhance (Du Pont, Paris, France) according to the instructions of the manufacturer and dried. Autoradiography was performed with Hyperfilm-MP (Amersham) at −80°C.

RNA was electrophoresed in 1.5% Agarose gels at 6 V/cm for 1 h. The electrophoretic buffer consisted of 89 m*M* Tris, 89 m*M* boric acid (pH 8.3) and 2 m*M* EDTA. The sample loading buffer also contained 1 *M* urea, 8.7% glycerol, 0.1%

SDS and 0.1% bromophenol blue. Ethidium bromide (2 μg/ml) in the gels was used for detection of the nucleic acids by UV light at 260 nm.

RESULTS

As the secondary structure of the RNAs influences the amount of bound ion-pairer, and thus the retention time of the molecules, it was imperative to destroy the base-paired regions by the addition of CH_3HgOH. As shown in Fig. 1, the amount of this additive is especially critical in the retention of the larger molecules, as exemplified by 18S and 28S rRNA. Without this compound, separation of these large RNAs is minimal (Fig. 1A), whereas with 3 m*M* CH_3HgOH the shorter retention times leave no separation window for the smaller molecules (Fig. 1B). Only at a concentration of 5 m*M* CH_3HgOH are the conditions met for the separation of the small and the larger RNAs (Fig. 1C). The complexation between CH_3HgOH and the RNA bases leads to a shift of the absorption maximum from 254 to 275 nm. All recordings were made at the latter

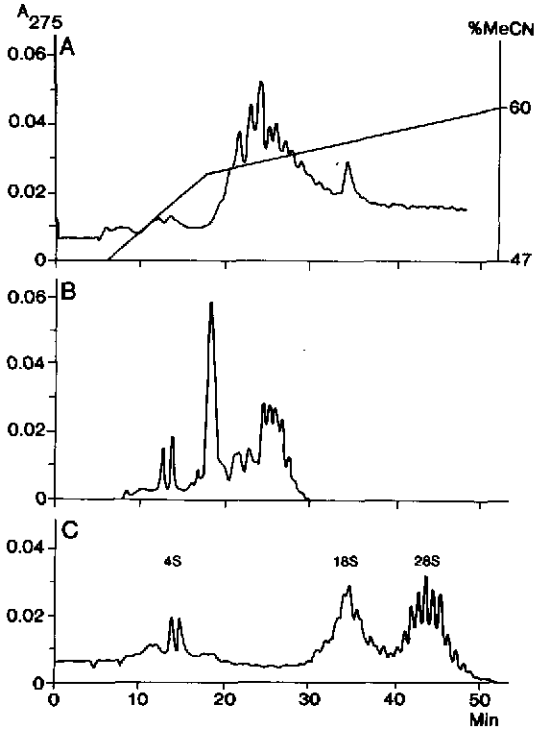

Fig. 1. Influence of methylmercuric hydroxide on the separation of total RNA isolated from reticulocytes: (A) without CH_3HgOH; (B) 3 m*M* CH_3HgOH; (C) 5 m*M* CH_3HgOH. Amounts of 48–55 μg of total RNA were used, column Nucleosil 300-5-C_4.

wavelength. Concomitantly the absorption coefficient of RNA increased two-fold. It was also found that CH_3HgOH was destroyed by H_3PO_4, which was used to keep the pH of the eluent within the working limits of the silica support. Sulphuric acid was used as a substitute, but as SO_4^{2-} ions are inhibitory in a translation system they had to be removed by dialysis.

The separation is further influenced by the concentration of the ion-pairing agent. Without ion-pairing the mRNAs were not retained on the column, with 2.5 m*M* TBA the separated rRNAs eluted close to the void volume; with 10 m*M* TBA the rRNAs needed high concentrations of acetonitrile for elution although the separation was rather poor. The best resolution was achieved with 5 m*M* TBA and this concentration was used in all the experiments. At the highest concentration of this compound in the translation system, i.e. 1.5 m*M*, it did not influence the translation of the mRNAs. Other ion-pairing compounds, such as tetrapropylammonium bromide and trimyristylammonium bromide, did not improve the separation of the mRNAs and led to losses of material, probably by strong interactions with the solid phase of the column (results not shown). When a support with longer hydrophobic chains (Zorbax C_8 column) was used with TBA as the ion-pairer, higher concentrations of acetonitrile were necessary to elute the RNAs with a concomitant deterioration of the resolution (results not shown). There probably exists a relation between the length of the hydrophobic chains of the support and of the ion-pairer for optimal resolution of the macromolecules.

Fig. 2 shows that, in order to recover minute amounts of mRNA from the

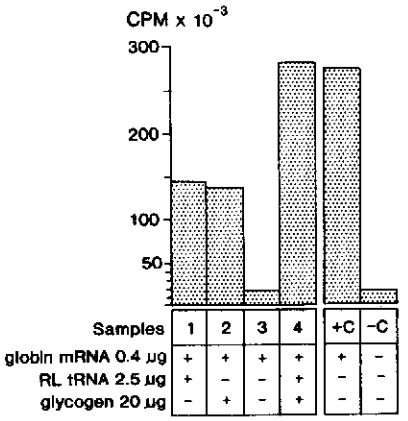

Fig. 2. Influence of carrier RNA and glycogen on the translation of recovered mRNA. To arrive at the highest percentage of acetonitrile (55%) used in the elution of RNAs, 400 µl of a mixture of solution A and B was prepared to which 0.4 µg of 9S globin mRNA was added. The RNA was ethanol-precipitated with or without rat liver tRNA and/or glycogen. The precipitates were taken up in 6 µl of water and dialysed. This RNA was then translated in 12.5 µl cell-free systems. +C: positive control of the same amount of RNA without the treatment mentioned, −C: negative control, *i.e.* translation of the system without added mRNA. Incorporation is given as cpm per 2-µl samples.

organic solutions, addition of carrier tRNA and glycogen is absolutely necessary. These molecules act as coprecipitators and do not influence the translation of the isolated mRNAs in the cell-free system used. At this stage, dialysis of the precipitated RNAs was effected to exchange the SO_4^{2-} ions and to lower the concentration of CH_3HgOH to 1 m*M*. At this concentration CH_3HgOH still inhibits the action of RNases. In the cell-free systems this concentration is further lowered by the addition of other components, and then CH_3HgOH does not show any influence on the incorporation of the systems. However, at concentrations above 1.5 m*M*, it progressively inhibits translation (results not shown).

Fig. 3 shows a typical separation of 19 μg of poly-A-enriched mRNA, together with the translational activity of these molecules in a cell-free system. The increasing amounts of component B result in a slow increase of acetronitrile and a decrease of the ion-pairing compound, which leads to the desorption pattern of the various RNAs. The small amount of injected mRNA still contains rRNA, as judged from the absorbance peaks around the 18S and 28S positions. Nevertheless, the translationally active RNAs give rise to a considerable incorporation of [^{35}S]methionine in the cell-free systems after separation into different species. Only half of the isolated RNA from each fraction was used for translation, and the incorporation depicted in Fig. 3 is only of small samples of the cell-free systems and thus represents the translational ability of *ca.* 8% of the total RNA in each fraction.

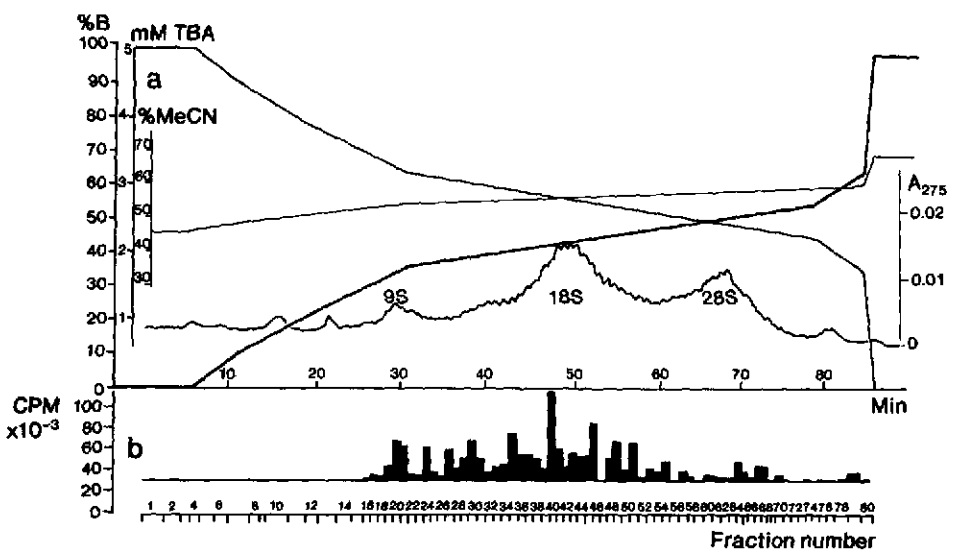

Fig. 3. Separation of 19 μg of poly-A-enriched rat liver RNA on a Nucleosil 1000-7 C_4 column. The various gradients are given. RNA from the fractions was precipitated in the presence of tRNA and glycogen, taken up in 10 μl of water and dialysed. Half of this RNA was translated in 12.5 μl cell-free systems, the other half was used for a determination of the molecular mass. The incorporation of the cell-free system is given as cpm per 2-μl sample. The incorporation without added mRNA was 33 000 cpm in a similar sample.

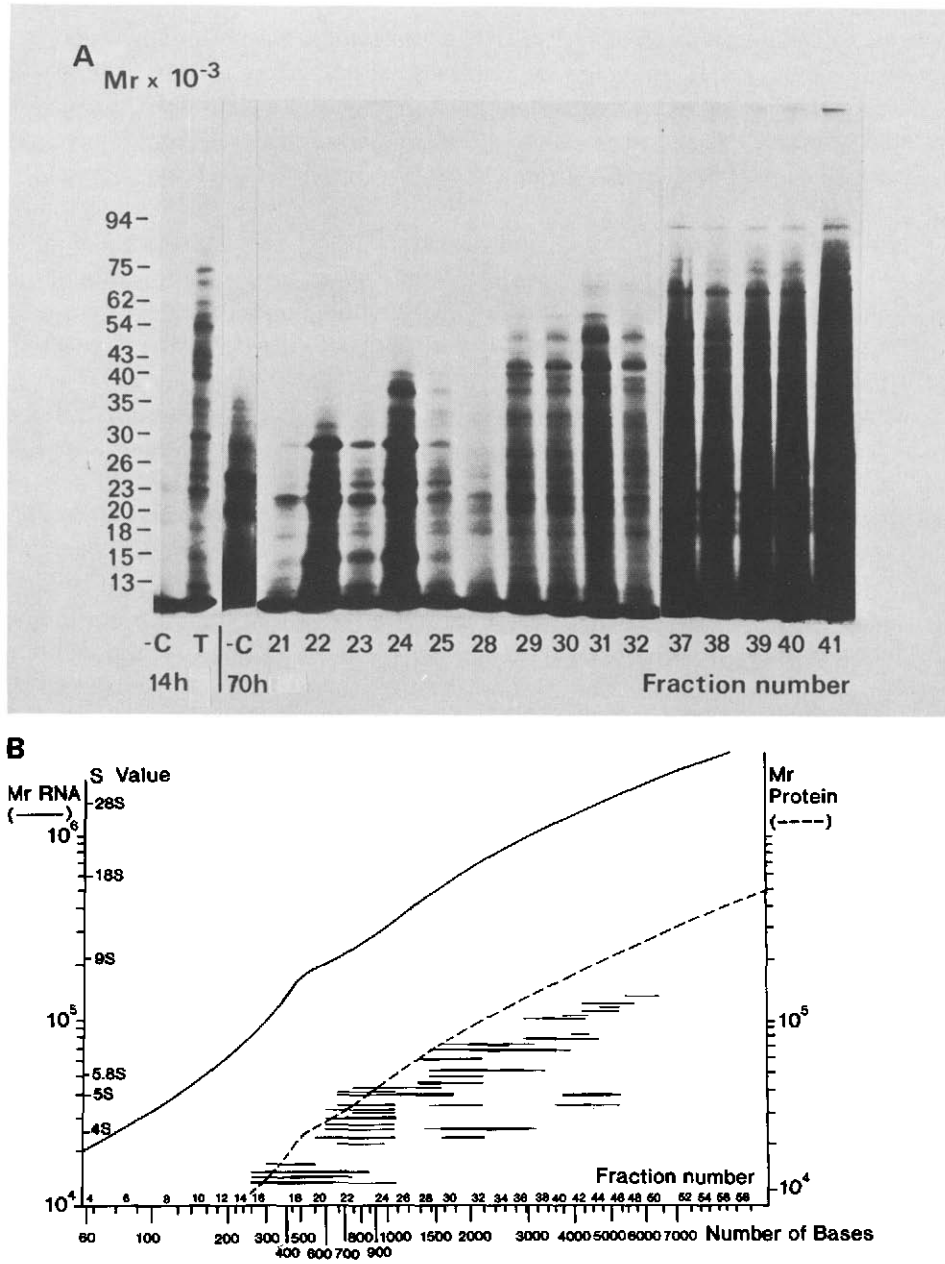

Fig. 4. (A) Fluorograph of the protein products translated from RNAs of some fractions isolated in the second experiment. Most RNAs elute in several fractions. The trend is for larger protein products from mRNAs, which elute at increased acetonitrile concentrations. The translational activity of the starting material is given in lane T. The exposure time of the film was 14 h for fractions 21–32 and 70 h for fractions 37–41. In lane C a control of the cell-free system without added mRNA is included for both exposure times. (B) Composite of the various fluorographs obtained in this second experiment. Translational products can be discerned in the range from 20 to nearly 200 kDa. The thick bars depict a relative abundance of a protein product, the thin lines minor amounts. Both ordinates are on a logarithmic scale. The S values on the RNA axis are the values of the marker RNAs used during electrophoresis (rRNA 28S, 18S, 5.8S and 5S; globin mRNA 9S and tRNA 4S). To arrive at the molecular masses given on the protein axis a standard set of proteins with known masses was included during electrophoresis.

In another experiment, 21 μg of poly-A-enriched RNA was separated, but the number of collected fractions was reduced to ensure higher concentrations of the minor mRNA species. The translational products were separated by gel electrophoresis, and the gels were fluorographed. A typical fluorograph from the second experiment mentioned is shown in Fig. 4A. Owing to the varying amounts of incorporation, the exposure time of the films had to be varied considerably in order to detect the minor translational products, and this led to overexposure of the products of the more abundant mRNAs. The negative control with an exposure time of 70 h shows a considerable incorporation of methionine, owing to contaminating mRNAs of the ribosomal subunits used in the translations. Only newly appearing proteins were scored as products of the isolated mRNAs. A consensus of the various film exposures of the experiment depicted in Fig. 4A and of other fractions is given in Fig. 4B. Half the amount of the RNAs from each fraction was electrophoresed on Agarose gels for a determination of their molecular masses. It can be seen that mRNAs up to 6000 bases, which give rise to proteins with molecular masses up to nearly $2 \cdot 10^5$ Da, can be separated. In some fractions (42–44, see Fig. 4B) protein products are encountered which would, according to their molecular mass, need only a small mRNA, although the elution sequence indicates mRNAs of high molecular mass. These mRNAs probably contain extensive untranslated regions, *i.e.* long leaders and trailers. However, most of the time the length of the mRNAs is directly related to the molecular masses of the ensuing proteins.

Fig. 5 is a composite of several experiments and shows the percentage of acetonitrile at which the various mRNAs elute *versus* the molecular masses of these molecules in the two column types used in this research. It is obvious that the highest resolution is obtained on supports with the larger pores.

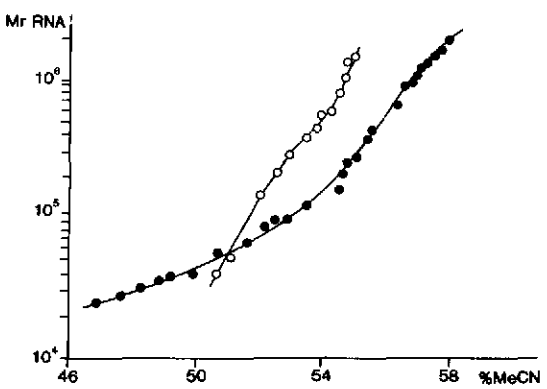

Fig. 5. Relation between the molecular mass of eluting RNAs and the percentage of acetonitrile. This figure is a composite of various experiments in which the RNAs were precipitated and run on Agarose gels to determine their molecular masses. (○) Nucleosil 300-5 C_4 column; (●) Nucleosil 1000-7 C_4 column.

DISCUSSION

The results clearly show a relation between the length of an mRNA and the percentage of the organic phase needed to release the molecules from the column material. Undoubtedly this organic phase leads to some unfolding of the secondary structures of the molecules, which accounts for some separation according to length, as mentioned by Nguyen *et al.* [17]. The addition of methylmercuric hydroxide, however, leads to complete unfolding with the concomitant increase of resolution achieved on the columns used. Furthermore, this compound is a powerful inhibitor of RNases and omittance of it leads to extensive breakdown of the mRNAs.

The inherent translational activity of the cell-free system gives rise to a set of small proteins that obscure the products of the smaller RNAs that we isolated. In the analysis of mRNAs coding for small proteins the wheatgerm system with its lower inherent activity can be very useful, but as the incorporation of this system is very sensitive to the inhibitory action of minute amounts of rRNA and CH_3HgOH it was impractical for our purposes.

The 'poly peak' phenomenon as mentioned by Garcia and Liautard [11,25] is also apparent in our experiments and is dependent on the slope increments of the organic phase. Decreasing the flow-rates alleviated this problem only in part, as the proportionately longer separation times impaired the resolution of mRNAs. The flow-rate chosen here gave the best resolution, but not the minimum of poly peaks.

It seems clear from the minimal amounts of starting material, of which a considerable part was still untranslatable RNA (rRNA), that the described separation method shows advantages. It might be especially useful when a cDNA of a specific mRNA needs to be synthesized where the availability of an enriched mRNA sample decreases the screening difficulties of the cloning procedure. If the primary structure of such an mRNA is known, a probe can be constructed that would facilitate the detection of the molecule.

ACKNOWLEDGEMENT

The isolation of the components of the cell-free systems by Marcelle Kasperaitis is gratefully acknowledged.

REFERENCES

1 G. K. McMaster and G. G. Carmichael, *Proc. Natl. Acad. Sci. U.S.A.*, 74 (1977) 4835.
2 W. Muller, *Eur. J. Biochem.*, 155 (1986) 203.
3 R. Hecker and D. Riesner, *J. Chromatogr.*, 418 (1987) 97.
4 M. Colpan and D. Riesner, *J. Chromatogr.*, 296 (1984) 339.
5 R. Hecker, M. Colpan and D. Riesner, *J. Chromatogr.*, 326 (1985) 251.
6 R. P. Singal, G. D. Griffin and G. D. Novelli, *Biochemistry*, 15 (1976) 5083.

7 R. P. Singal, *J. Chromatogr.*, 266 (1983) 359.
8 J. D. Pearson and F. E. Regnier, *J. Chromatogr.*, 255 (1983) 137.
9 R. R. Drager and F. E. Regnier, *Anal. Biochem.*, 145 (1985) 47.
10 M. H. Simonian and M. W. Capp, *J. Chromatogr.*, 266 (1983) 351.
11 S. Garcia and J. P. Liautard, *J. Chromatogr.*, 296 (1984) 355.
12 N. E. Hoffman and J. C. Liao, *Anal. Chem.*, 49 (1977) 2231.
13 P. A. Perrone and P. R. Brown, *J. Chromatogr.*, 317 (1984) 301.
14 H. Schott, R. Semmler and H. Eckstein, *J. Chromatogr.*, 389 (1987) 165.
15 K. Makino, H. Ozaki, T. Matsumoto, H. Imaishi, T. Takeuchi and T. Fukui, *J. Chromatogr.*, 400 (1987) 271.
16 D. A. Usher, *Nucleic Acids Res.*, 6 (1979) 2289.
17 P. N. Nguyen, J. L. Bradley and P. M. McQuire, *J. Chromatogr.*, 236 (1982) 508.
18 R. B. Simpson, *J. Am. Chem. Soc.*, 86 (1961) 2059.
19 J. M. Bailey and N. Davidson, *Anal. Biochem.*, 70 (1976) 75.
20 P. M. Lizardi, *Methods Enzymol.*, 96 (1983) 24.
21 H. Aviv and P. Leder, *Proc. Natl. Acad. Sci. U.S.A.*, 69 (1972) 1408.
22 A. K. Falvey and T. Stachelin, *J. Mol. Biol.*, 53 (1970) 1.
23 M. H. Schreier and T. Staehelin, in E. K. F. Bautz (Editor), *24th Mosbach Colloquium*, Springer Verlag, Heidelberg, 1973, p. 335.
24 U. K. Laemmli, *Nature*, 227 (1970) 680.
25 S. Garcia and J. P. Liautard, *J. Chromatogr. Sci.*, 21 (1983) 398.