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Efficient recovery of functionally intact mRNA from agarose gels via transfer to an ion-exchange membrane

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ABSTRACT

A simple method is described for the efficient recovery of intact mRNA from high resolution agarose gels. Fractionation of RNA is accomplished by gel electrophoresis under denaturing conditions using methylmercuric hydroxide. The RNA in the gel is then transferred electrophoretically to a diethylaminoethyl (DEAE)-membrane. After reversing the methylmercuric modification of the RNA, the membrane is sliced into narrow sections and the RNA is eluted at 65° with a high ionic strength buffer containing 6M guanidine hydrochloride. RNA isolated by this procedure is suitable for subsequent enzymatic reactions, including *in vitro* translation and reverse transcription. The major advantages offered by this procedure are: 1) The membrane-bound RNA is a replica of the high resolution fractionation pattern achieved in the gel. 2) The immobilization and concentration of RNA and the removal of gel matrix contaminants are all accomplished in one step. 3) Small quantities of RNA are efficiently recovered and are suitable for subsequent biochemical manipulations.

The method is of general utility for any biological system. We have applied its use to the fractionation, recovery, and analysis of mRNA from *Xenopus* liver and have identified cDNA clones complementary to albumin mRNA.

INTRODUCTION

Experimentally convenient techniques for high resolution fractionation of nucleic acids are essential tools for current investigations of genome organization and expression at the molecular level. Gel electrophoresis is the method of choice for separating DNA and RNA molecules with maximum resolution for both analytical and preparative purposes. In analytical investigations, fractionated nucleic acids are frequently transferred out of a gel and immobilized on a solid support surface. Southern (1) first applied this approach to the transfer of DNA from agarose gels to nitrocellulose membranes. Alwine et al. (2) developed techniques for the transfer and binding of RNA to chemically activated paper. By these procedures a replicate pattern of the nucleic acids in the gel is obtained and the immobilized molecules are accessible to analysis using highly sensitive molecular hybridization techniques.

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For the preparative isolation of nucleic acids from gels, it is of importance to obtain efficient recovery of material which is sufficiently pure for subsequent use in enzymatic reactions. In the past, several approaches have been taken in an effort to achieve these goals. These methods fall into four categories: electrophoretic elution, gel compression, gel dissolution, and diffusion (3). Each of these procedures has been used successfully for recovery of DNA, although no one method is entirely satisfactory for all purposes. Less success has been achieved for recovery of RNA, which is generally more sensitive to degradation and prone to artifacts due to secondary structure.

Regardless of the particular technique employed, a general problem is that agarose gel material frequently contaminates the recovered nucleic acids and inhibits subsequent enzymatic reactions. Therefore additional purification steps are usually required. Ion-exchange chromatography on diethylaminoethyl (DEAE)-cellulose is a very effective procedure commonly employed for removal of these contaminants. Unfortunately, these additional steps usually result in low overall recovery of the nucleic acids and/or laborious manipulations involving many samples.

In the present communication we describe a method in which the concept of gel-to-membrane transfer has been applied to the preparative isolation of nucleic acids. By combining the transfer technique with the principle of ion-exchange chromatography, the recovery and purification steps are united into one operation. Hence this procedure allows rapid and efficient recovery of virtually any RNA from agarose gels. The ion-exchange support used here is a commercially available DEAE-membrane, on which DNA or RNA can be transiently rather than permanently bound. The DEAE-membrane is relatively strong and does not disintegrate when wet. It is therefore far more useful than ion-exchange cellulose sheets which have also been used to bind nucleic acids transferred from gels, but which are fragile and very difficult to keep intact (4-7). Because the DEAE-membrane can be sliced into extremely narrow, yet easily manipulated sections, this recovery procedure preserves the high resolution fractionation of the RNA achieved in the gel to a far greater extent than does any of the methods mentioned above.

EXPERIMENTAL PROCEDURES

Purification and End-Labeling of RNA

Total cellular RNA was prepared from the livers of female *Xenopus* following the procedure of Deeley et al. (8) except that guanidine-HCl was

used at 7M and the ethanol and sodium acetate precipitations were extended to 12-24 hrs. Poly(A)$^+$RNA was selected by hybridization to oligo(dT)-cellulose as described (9), with the inclusion of 0.1mM EDTA pH 8 in the binding and elution buffers. The eluate was adjusted to 0.2M potassium acetate pH 5.2. The RNA was precipitated by addition of two volumes of ethanol, washed twice with 70% ethanol, and resuspended in H_2O.

^{32}P-labeled RNA was prepared by 5'-end labeling following procedure 5B of Maxam and Gilbert (10). Total cellular RNA, without pretreatment with alkaline phosphatase, was the substrate in this reaction. The reaction mixture was extracted with chloroform:isoamyl alcohol and Sephadex G50 chromatography was performed in 0.3XE buffer (see below).

Methylmercuric Hydroxide Agarose Gel Electrophoresis

Electrophoretic separation of RNA was based on the procedure of Bailey and Davidson (11). All handling of methylmercuric hydroxide (CH_3HgOH) was carried out in a fume hood. The fractionation procedure described below was used in order to achieve very high resolution, since under these conditions RNA migrated in the gel as narrow straight bands. Methylmercuric hydroxide agarose gels run by more conventional procedures are equally suitable for the subsequent steps in the method. Vertical slab gels (17x20x0.5 cm) containing 1% agarose (Seakem) and 5mM CH_3HgOH (Alfa) were cast using a comb that formed wells narrower than the thickness of the gel so that there was agarose around all four sides of each well. The buffer used in the gel and electrode reservoirs was a 3:10 dilution of E buffer which is .05M boric acid, .005M $Na_2B_4O_7 \cdot 10H_2O$, .01M sodium sulfate, .001M Na_2EDTA, pH 8.19 (11). Samples were loaded on the gel in molten agarose as follows. A 5X stock solution of agarose slurry containing 0.5% agarose, 25% glycerol, 0.05% Bromphenol Blue was prepared as described (12). An aliquot of this solution was boiled and held at 65°. RNA samples in 15µl were brought to 65°, 5µl of the agarose solution was added, and immediately before loading 5µl of 50mM CH_3HgOH, 1.5XE buffer at 22° was added. Electrophoresis was carried out at approximately 100V for 4 hrs with recirculation of the electrode buffer.

DNA fragments of known size were obtained from pBR322 DNA by digestion with *Hind*III and codigestion with *Hind*III and one of the following: *Ava*I, *Pvu*II, or *Pst*I. Restriction enzymes were from New England Biolabs or Bethesda Research Labs. For defined sequence RNA size markers we used *E. coli* ribosomal RNAs (Boehringer) and the *Xenopus* 18S RNA present in our preparation of liver RNA. The sizes of these molecules are 2904 bases for 23S (13), 1825 for 18S (14), and 1541 for 16S (15).

To analyze nucleic acids within the gel, the gel was first soaked in 0.5M ammonium acetate 2 X 30 min. ^{32}P-RNA was detected by drying the gel onto heavy paper (Hoefer) under vacuum, 1 hr without heat and 2 hrs with heat, and exposing Kodak XAR5 film at -40°. Unlabeled molecules were stained with ethidium bromide and photographed as described below.

Electrophoretic Transfer to and Elution from DEAE-Membrane

DEAE-membrane (Schleicher & Schuell, #NA-45) was first wet in H_2O. It was then treated with 0.5M NaOH for 1 min, followed by thorough rinsing with H_2O as recommended by the manufacturer. The membrane was then soaked in elution buffer (see below) at 65° for 15 min. After rinsing with H_2O the membrane was soaked in 0.3XE buffer and used within 15 min.

Electrophoretic transfer was carried out in a Trans-Blot Cell (Bio-Rad). Immediately following electrophoretic separation of RNAs, the gel was placed on 2 pieces moist 3MM paper (Whatman). The prepared DEAE-membrane was positioned on the gel and gently rubbed to ensure good contact. Two pieces of moist 3MM were placed on top of the membrane. This was mounted in the gel holder with 3 pre-wet sponge pads and a rubber band was placed lengthwise around the middle. The DEAE-membrane was placed anodal to the gel. The transfer buffer, 0.3XE buffer, was stirred and was maintained at 4° by circulation of externally cooled fluid through the cooling coil. Transfer was carried out in a fume hood because CH_3HgOH was still present in the gel. A voltage of 30V was applied and for convenience the transfer was allowed to continue overnight (up to 24 hrs). A high amperage power supply was not required since under these conditions the current was less than 200mA. For the detection of membrane-bound ^{32}P-RNA, the membrane was soaked in ammonium acetate as described below and dried onto paper under vacuum for 30 min with heat in order to avoid shriveling.

To remove the methylmercuric moiety from the RNA, the DEAE-membrane was soaked in 20mM ammonium acetate 3 X 20 min. The membrane was then placed on moist 540 paper (Whatman) and sliced with a razor blade. Each section was cut to fit on the bottom of a polypropylene vial with a 1.1 cm diameter (Rochester Scientific).

RNA was eluted from the membrane sections by incubation for 10 min at 65° in 200µl 6M guanidine-HCl (Schwartz/Mann, ultra-pure), 17mM EDTA, 17mM sodium acetate, 50µg/ml deacylated (16) tRNA, pH 6.5. A second elution was carried out with an additional 200µl of eluant. The RNA in the combined eluates was precipitated by the addition of 2 volumes of ethanol and stored at -40° overnight. A total of three ethanol precipitations were performed,

each time dissolving the RNA in 200μl H_2O and adjusting to 0.2M potassium acetate pH 5.2. After the first ethanol precipitation, the redissolved RNA was extracted once with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and once with chloroform:isoamyl alcohol (24:1). The organic extractions were not absolutely required for the recovery of translation activity, but they aided in the removal of particulate matter which often formed after the first ethanol precipitation. After the second ethanol precipitation the RNA was extracted twice with ether. The final RNA pellet was washed twice with 70% ethanol, dissolved in H_2O, and stored at -80°.

<u>In Vitro Translation and Protein Gel Electrophoresis</u>

RNA was translated in a wheat germ system (17). Each 25μl reaction contained: 5μl wheat germ extract (treated with micrococcal nuclease (18)), 24mM Hepes-KOH pH 7.6, 120mM potassium acetate, 2mM magnesium acetate, 1mM ATP, 0.2mM GTP, 0.6mM spermidine, 2mM dithiothreitol, 8mM creatine phosphate, 40μg/ml creatine phosphokinase, 40μg/ml deacylated (16) calf liver tRNA (Boehringer), 25μM each amino acid except methionine, 10μCi [^{35}S]methionine (1100 Ci/mmole, New England Nuclear). Incubations were at 22° for 2 hrs. Trichloroacetic acid (TCA) precipitation was carried out by spotting 2μl aliquots on 3MM filters (Whatman). The filters were incubated in ice cold 10% TCA for 60 sec, boiled in 5% TCA for 10 min, rinsed with ethanol, dried thoroughly, and counted in 0.5% 2,5-diphenyloxazole (PPO)/ 0.01% p-bis-[2-(5-phenyloxazolyl)] benzene (POPOP) in toluene.

SDS-polyacrylamide gel electrophoresis was carried out using 9-15% exponential gradient gels as described (19). For autoradiography preflashed (20) Kodak XAR5 film was exposed at -40°. A Joyce-Loebl Microdensitometer was used for analysis of autoradiograms.

<u>Reverse Transcription and Molecular Hybridization</u>

Synthesis of cDNA was carried out at 44° for 30 min in the following 20μl reaction mixture (21): 50mM Tris-HCl (1M stock pH 8.7 at 22°), 10mM $MgCl_2$, 30mM β-mercaptoethanol, 10μg/ml oligo$(dT)_{12-18}$ (Collaborative Research), 1mM each ATP, CTP, and GTP, 2.5 μM TTP, 30μCi [α-^{32}P]TTP (2800 Ci/mmole, New England Nuclear), 8 units AMV reverse transcriptase (65,000 units/mg, from J.W. Beard, Life Sciences, St. Petersburg, FA). The reaction was terminated by adjustment to 20mM EDTA, 0.1% SDS and 10μg carrier tRNA was added. The sample was extracted with chloroform:isoamyl alcohol and chromatographed on Sephadex G50 in 50mM NaCl, 10mM Tris-HCl pH 8, 1mM EDTA.

To prepare plasmid DNA molecules for gel electrophoresis, cells were lysed by the procedure of G. Bell (personal communication). Colonies were

transferred from agar plates with a toothpick into 20μl 10mM Tris-HCl pH 8. Following freezing and thawing, 3μl of 0.25M EDTA pH 8:10mg/ml RNase A (Sigma):5mg/ml lysozyme (Sigma) (1:1:1) was added and incubation was for 30 min at 4°. Five μl 50% glycerol, 1% SDS, 0.05% Bromphenol Blue was added, followed by incubation for 10 min at 65° and vigorous vortexing for 10 sec. The entire sample was loaded on a gel of 1% agarose and electrophoresis in Tris-acetate buffer (22) was performed for 450 V·hrs. The gel was treated with 50mM NaOH for 60 min so that RNA would not appear in the photograph. The gel was neutralized by soaking in 0.5M ammonium acetate, stained with 1μg/ml ethidium bromide in ammonium acetate, destained, and photographed over an ultraviolet light (254 nm) through a yellow filter on Polaroid film (665). To facilitate the transfer of DNA out of the gel, the DNA was nicked by soaking the gel in 0.25M HCl for 5 min (23). Transfer to a nitrocellulose membrane was carried out as described by Southern (1).

The nitrocellulose filter was pretreated for 18 hrs at 37° with 50% formamide (MCB, adsorbed for 30 min with Bio-Rad AG501-X8(D)), 3XSSC, 10mM Hepes-NaOH pH 7.5, 0.1% SDS, 1mM EDTA, 25μg/ml E. coli DNA, 0.1% crystalline bovine serum albumin (Calbiochem), 0.1% polyvinylpyrrolidone, 0.1% Ficoll (24). Hybridization was carried out for 24 hrs at 37° in a solution of the same composition plus ^{32}P-cDNA (2.5 X 10^6 cpm) which was denatured together with 500μg E. coli DNA by boiling for 2 min in 0.3M NaOH and neutralized with 0.3M HCl. The filter was washed at 22° in 2XSSC, 0.1% SDS 2 X 15 min and at 50° in 0.1XSSC, 0.1% SDS 2 X 45 min and air dried. Preflashed Kodak XAR5 film was exposed at -40° with an intensifying screen (Dupont).

cDNA Cloning

Our cDNA clone library representing mRNA from normal frog liver was constructed by cloning mRNA:cDNA hybrids as described by Zain et al. (25).

RESULTS AND DISCUSSION

Denaturation and Electrophoresis of RNA in Methylmercuric Hydroxide

Two features of critical importance to the method described here are the high resolution fractionation of RNA and the recovery of the RNA in a functionally intact state. Fully denaturing conditions must be used during electrophoresis in order to achieve maximum resolution of RNA. The subsequent recovery of biological activity requires that the RNA not be permanently modified by the treatment. Both of these criteria are met by the denaturing agent methylmercuric hydroxide, which has been shown to be a strong yet readily reversible denaturant (11). Thus we have employed a

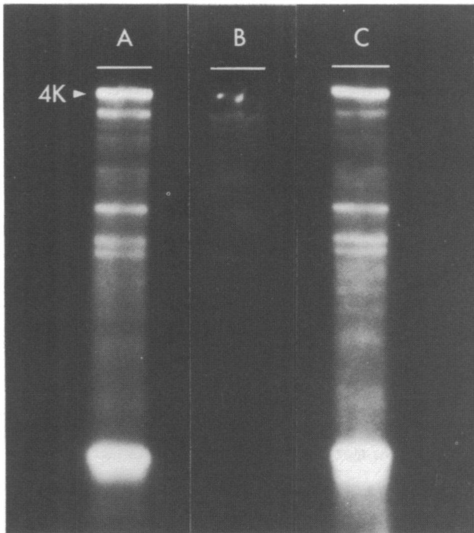


Figure 1. Electrophoretic Separation of RNA in CH_3HgOH-Agarose Gel and Transfer to DEAE-Membrane. (A) 50ng ^{32}P-RNA (6 X 10^5 cpm/μg) resolved in a CH_3HgOH-agarose gel (100V for 4 hrs). (B) ^{32}P-RNA remaining in gel after electrophoretic transfer of sample shown in (C). (C) DEAE-membrane bound RNA after electrophoretic transfer of 50ng ^{32}P-RNA from gel. The figure is the negative image of an autoradiogram. The size markers were identical to those used for Figure 3.

slightly modified technique of methylmercuric hydroxide agarose gel electrophoresis for the fractionation of RNA. Radioactively labeled total RNA from *Xenopus* liver resolved in this manner is shown in Figure 1A.

Electrophoretic Transfer to the DEAE-Membrane

The next step in the procedure is transfer of RNA from the gel to a DEAE-membrane. The efficiency of this process was assessed by following the fate of radioactive RNA. After electrophoretic transfer was performed as described in Experimental Procedures, only a small amount of RNA remained in the gel (Figure 1B). The pattern of ^{32}P-labeled RNA bound to the DEAE-membrane (Figure 1C) was a replica of that in the gel. As evidenced by these results, the efficiency of transfer was nearly 100% for RNAs up to approximately 4000 nucleotides in length, the largest molecules in this sample.

In order to restore the biological activity of the RNA, the methylmercuric moiety must be removed. Ammonium acetate has been shown to be a suitable reagent for accomplishing this reversal (11). For the experiments described here the RNA was transferred out of the gel immediately after fractionation. Then the membrane with bound RNA was soaked in 20mM ammonium acetate. No detectable loss of RNA from the membrane occurred at this step (data not shown).

An alternative approach for achieving reversal of the methylmercuric modification is soaking the gel itself in ammonium acetate prior to

electrophoretic transfer. An advantage of this procedure is that, since the methylmercuric hydroxide has been removed, it is no longer necessary to perform the electrophoretic transfer in a fume hood. There are, however, disadvantages to this approach. Diffusion and loss of small RNA molecules occurs during the soaking. Comparison of the samples in Figure 1 reveals that the membrane-bound RNA (Figure 1C) included more of the smaller species of RNA than did the RNA sample in the gel (Figure 1A) since it was necessary to soak the gel section in ammonium acetate in preparation for autoradiography. For some applications, loss of smaller RNAs may be acceptable. In addition, we found that soaking the gel in 0.5M ammonium acetate (11) was unsuitable because it reduced the efficiency of transfer of RNA out of the gel to approximately 50%. Pretreatment of the gel with lower concentrations of ammonium acetate might permit more efficient transfer.

Another possible variation in our procedure is transferring the RNA from the gel to the DEAE-membrane by blotting procedures (2, 26) rather than by electrophoresis. We have employed electrophoretic transfer because it is more rapid and efficient than blotting, particularly for large RNA molecules (27, 28).

Optimization of Elution of RNA from the DEAE-Membrane

RNA was eluted from the DEAE-membrane at 65° with a solution containing 6M guanidine-HCl, 17mM EDTA, 17mM sodium acetate, 50μg/ml tRNA, pH 6.5, as described in Experimental Procedures. This protocol was chosen after examination of several factors which affect overall recovery of RNA, including eluant composition and concentration, length of the RNA molecules, and elution temperature. These conditions represent the optimum for recovery of functionally intact large RNA molecules in high yield.

We investigated guanidine-HCl as a possible eluant because it is an organic salt which interrupts ionic interactions between nucleic acids and proteins (29). NaCl was also tested since it is commonly used to recover nucleic acids bound to DEAE-cellulose (5, 7). We compared the relative efficiencies of these two salts in eluting RNA molecules which ranged in size from less than 500 nucleotides up to approximately 4000 nucleotides. To do this, ^{32}P-labeled RNA was separated by size in a methylmercuric hydroxide agarose gel and transferred electrophoretically to a DEAE-membrane. Parallel strips of the DEAE-membrane containing identical RNA samples were either left untreated (Figure 2A) or were eluted at 65° with 6.6M guanidine-HCl (Figure 2B). Guanidine-HCl eluted all RNAs up to 4000 nucleotides in length, the largest size in this sample, with virtually

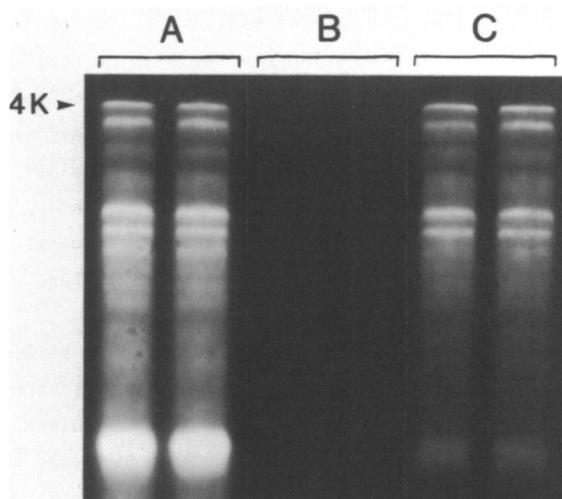


Figure 2. Elution of Size-Fractionated RNA from DEAE-Membrane with Guanidine-HCl or NaCl.

^{32}P-labeled RNA was separated by size in a CH_3HgOH-agarose gel, electrophoretically transferred to a DEAE-membrane, and treated with ammonium acetate. Strips with identical RNA samples were incubated as described below and processed for autoradiography. The data are shown in a negative image of the autoradiogram. (A) No Elution. (B) Elution with 6.6M guanidine-HCl, 19mM sodium acetate, pH 7 at 65°, 2 X 10 min. (C) Elution with 1M NaCl, 10mM Tris-HCl pH 7.5 at 65°, 2 X 10 min.

100% efficiency. In contrast, elution efficiency with 1M NaCl decreased dramatically with increasing size of the RNA, with no detectable elution of the largest RNA molecules (Figure 2C). Higher concentrations of NaCl, up to 5M, were no more effective than 1M NaCl (data not shown).

Optimization of the elution conditions included an investigation of the elution efficiency as a function of two variables, the molar concentration of guanidine-HCl, and the temperature of elution. Concentrations of guanidine-HCl between 1.0M and 6.6M were tested and the effect of temperature over the range 37° to 65° was examined. We found that maximal removal of RNA from the membrane required at least 6M guanidine-HCl and a temperature of 65° (data not shown). Lowering either the salt concentration or the temperature resulted in a sharp decrease in efficiency of recovery or RNA, particularly the largest RNA molecules.

Stability of RNA During Elution

Recovered RNA must be both chemically and functionally intact to be maximally useful in molecular investigations. The extent of degradation of

RNA was measured by exposing ^{32}P-labeled RNA to the elution conditions and then fractionating the RNA in a gel in parallel with an equal aliquot of untreated RNA. Because large RNA molecules are statistically more likely to be cleaved, these molecules provide the most sensitive test for degradation. Therefore the amount of the 4000-nucleotide RNA in each sample was quantitated by densitometric scanning of the autoradiogram. Approximately 60% of these molecules remained intact under the experimental conditions (data not shown). Among smaller RNA species, the percentage of intact molecules recovered was even greater.

In an effort to find elution conditions which would be less deleterious to the RNA, we explored the use of 2M triethylammonium bicarbonate, a volatile salt used to elute nucleic acids from DEAE-cellulose (4). This salt was nearly as effective as 6M guanidine-HCl in removing RNA from the membrane. However, using fresh commercially available triethylamine adjusted to pH 8 with CO_2, we found that more degradation of the RNA occurred than with 6M guanidine-HCl. In addition, triethylammonium bicarbonate treatment resulted in inhibition of the *in vitro* translation reaction. It is possible that more highly purified triethylamine would be less deleterious to the RNA.

In conclusion, the elution conditions which we ultimately adopted represent a balance between three important factors: 1) efficient elution of RNA from the membrane, 2) molecular integrity of the RNA, and 3) ease of preparation and handling of the eluant. Ideally it would be most desirable to have no degradation of the RNA during the elution. Nonetheless a recovery of 60% or more intact molecules allows the analysis of the RNA by methods, such as *in vitro* translation, which require full-length molecules.

Enzymatic Reactions Using Recovered RNA

In order to demonstrate the recovery of functionally intact RNA by our procedure, we have fractionated *Xenopus* liver mRNA and used it for *in vitro* translation and reverse transcription. The mRNA was first resolved in a methylmercuric hydroxide agarose gel. Nucleic acid markers of defined sequence were run in a separate lane of the same gel and their distance of migration is shown in Figure 3A. The RNA was then electrophoretically transferred from the gel to a strip of DEAE-membrane. Removal of the methylmercuric moiety was accomplished by treatment with ammonium acetate, the membrane was cut into sections 3mm in width, and the RNA was eluted from the individual sections.

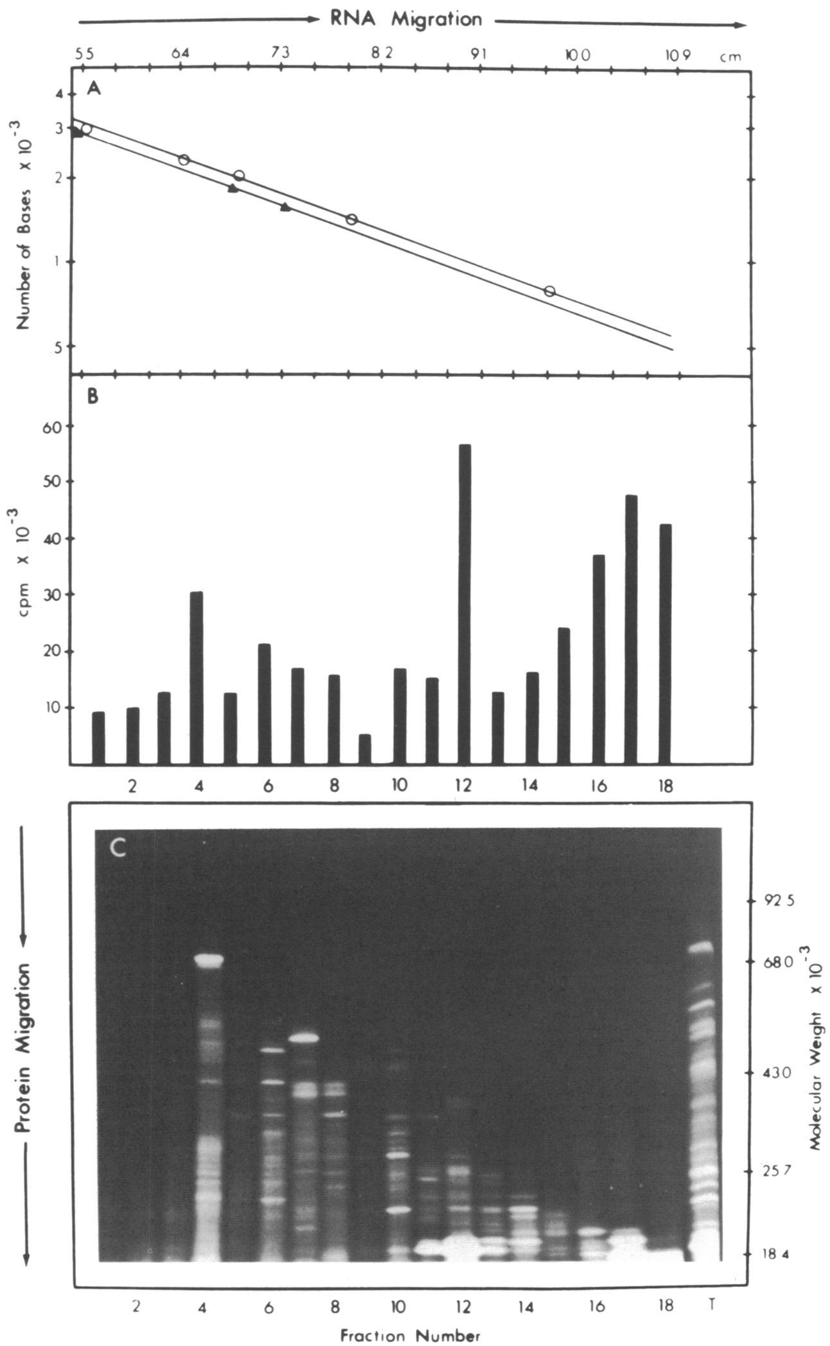
In Vitro Translation: The functional activity of the RNA in each

fraction was characterized by translation *in vitro*. This is a stringent test of molecular integrity since it requires undegraded mRNA for synthesis of complete polypeptide chains. We have employed a wheat germ *in vitro* translation system because it provides a clearer pattern of the particular translation products of interest to us, but similar results were obtained with a rabbit reticulosyte system. For each fraction, the amount of ^{35}S-methionine incorporated into protein was determined by acid precipitation (Figure 3B) and the translated polypeptides were displayed in a SDS-polyacrylamide gel (Figure 3C).

Several general conclusions are immediately evident from these data. First, the total set of translation products was distributed into several fractions, eighteen in this particular experiment. Second, the average molecular weight of the protein products in each fraction gradually decreased in parallel with the decreasing size of the fractionated RNA. And third, the overall pattern of translation products in the fractions closely resembled that of unfractionated RNA. These results support our conclusion that the majority of messenger RNAs remained intact during the preparative isolation.

The pattern of bands in Figure 3C also provides information about the resolving power of our fractionation and recovery method. In several instances a particular translation product occurred in a single fraction, and was not present in neighboring fractions. A clear example of such a case is the largest polypeptide in fraction 4. This translation product is the precursor to albumin (unpublished experiments). Other examples can be seen in fractions 6, 7, and 10. These results reflect the precision of the recovery procedure in preserving the high resolution fractionation of the RNA achieved in the gel. For the purposes of demonstration here, we cut the membrane into sections 3mm in width so that the translation products of the entire spectrum of mRNAs could be displayed in a single gel. Alternatively, the whole DEAE-membrane strip, or a smaller portion of interest, can be cut into narrower sections to achieve even higher resolution of individual RNA species. In an experiment designed for maximum resolution of less abundant mRNAs, we sliced the membrane into sections about 0.5mm in width, using a mechanical chopping device, and then translated the RNA in each fraction. Under those conditions albumin mRNA was separated into several neighboring fractions. In many cases, two mRNAs which had been recovered from a single 3mm membrane section were now clearly separated into adjacent fractions.

In addition, the data in Figure 3C allow an estimate to be made of the


RNA Migration
55
64
73
82
91
100
109
cm
A
Number of Bases x 10-3
B
cpm x 10-3
C
Protein Migration
Molecular Weight x 10-3
92.5
68.0
43.0
25.7
18.4
Fraction Number
T

relative amounts of translated and untranslated sequences in a particular messenger RNA. For example, a striking case of a mRNA with a large untranslated region can be seen in fraction 12. The major translation product in this lane has a molecular weight of approximately 18,000, which could be coded for in about 500 nucleotides, whereas the mRNA in this fraction is about 1000 bases in length. This discrepancy suggests that the mRNA for this protein has an untranslated region of about 500 nucleotides. Two other specific examples are the translation products of two of the subunits of fibrinogen, which we have characterized in detail (19, 30). Although these polypeptides are smaller than albumin, they are coded by mRNAs larger than albumin mRNA (experiments in progress).

Two other factors, premature translation termination in the wheat germ system and partial degradation of the RNA during elution from the DEAE-membrane, could account for the presence of smaller polypeptides in particular fractions. It is not clear, however, to what extent such events would result in discrete bands rather than a background distribution of smaller translation products.

Reverse Transcription and Hybridization to cDNA Clones: In order for RNA eluted from the DEAE-membrane to be characterized further, it is necessary that it be usable in other enzymatic reactions. We show here that it is a suitable substrate for reverse transcription. The resulting cDNA can be used either as a hybridization probe or to synthesize and clone double stranded cDNA. The RNA in fraction 4 of the experiment shown in Figure 3C was chosen for cDNA synthesis because it was highly enriched for albumin mRNA. The RNA recovered in this fraction generated approximately 10^7 cpm of ^{32}P-labeled cDNA under the specific reaction conditions described in Experimental Procedures.

Figure 3. *In Vitro* Translation of *Xenopus* liver mRNA after Fractionation in CH_3HgOH Gel and Recovery from DEAE-Membrane.

Approximately 10μg *Xenopus* poly(A)$^+$RNA was resolved by CH_3HgOH-agarose gel electrophoresis (90V for 4 hrs). (A) shows the distance of migration of DNA (O) and RNA (▲) markers (see Experimental Procedures) run in parallel lanes of the same gel. The mRNA was transferred from the gel to a DEAE-membrane and eluted from sections (3mm in width) as described in Experimental Procedures. One-half of the material eluted from each section was used in an *in vitro* translation reaction of 25μl. (B) shows the amount of incorporation of ^{35}methionine into protein in a 2μl aliquot from the translation reactions. (C) shows the electrophoretic analysis of the polypeptides in a 10μl aliquot from each *in vitro* translation reaction. Lane T shows the translation products from unfractionated *Xenopus* liver mRNA. These data are represented in a negative image of an autoradiogram.

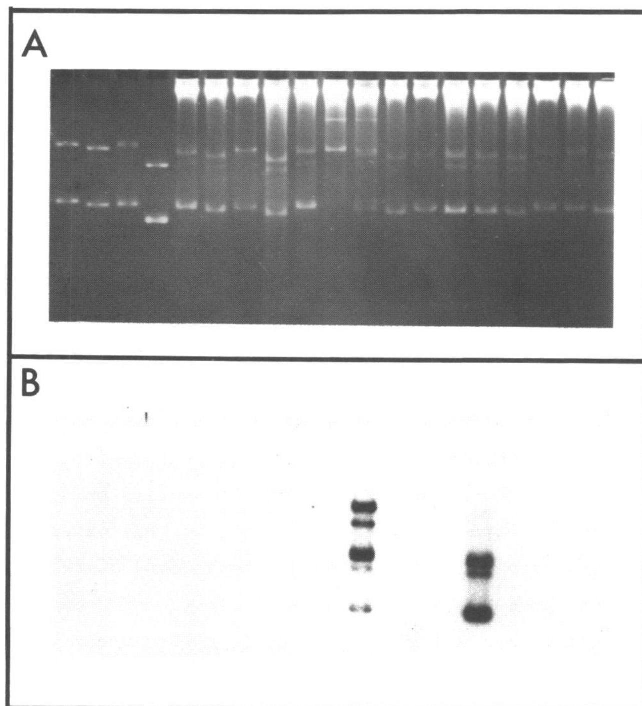


Figure 4. Reverse Transcription of Recovered RNA and Hybridization to cDNA Clones.

(A) Plasmid DNAs from several individual cDNA clones complementary to Xenopus liver mRNA were resolved by gel electrophoresis and stained with ethidium bromide. (B) The DNA was transferred to a nitrocellulose membrane and hybridized with ^{32}P-labeled cDNA synthesized from one-fourth of the RNA recovered in fraction 4 (Figure 3).

We had previously prepared a cDNA library of sequences complementary to total mRNA from Xenopus liver. In order to screen this library with the radioactive probe made from fractionated RNA, the DNA from several of the cDNA clones was resolved by gel electrophoresis. The visualization of this DNA by staining with ethidium bromide is shown in Figure 4A. The DNA was transferred from the gel to a nitrocellulose membrane and hybridized with the radioactively labeled cDNA. Figure 4B shows two clones among this set which hybridize with the probe. Thus these clones are very likely candidates for cDNA sequences complementary to albumin mRNA. We have subsequently confirmed that at least one of these clones is indeed albumin cDNA. This was shown by comparison of the restriction enzyme digestion pattern with published maps (31).

Estimate of Overall Efficiency

The overall efficiency of our recovery procedure was determined by comparing the amount of fractionated and unfractionated RNA required to synthesize equal amounts of any single in vitro translation product. Such an analysis provides a conservative estimate of yield since it scores only full-length RNA molecules. For this calculation we used the albumin translation product, the largest major polypeptide at approximately 70K daltons in fraction 4 (Figure 3C). The intensity of the albumin band translated from 0.2μg of unfractionated polyA$^+$ RNA was equivalent to the intensity of the same band translated from the RNA recovered after fractionation of 2.0μg. Thus a yield of approximately 10% was achieved in the experiment shown here. In a subsequent experiment, the yield of the albumin translation product was approximately 20%, as determined by the same analysis. This level of recovery allows for the isolation and analysis of RNA species of low abundance.

CONCLUDING REMARKS

We have developed a simple and efficient procedure for the recovery of RNA from methylmercuric hydroxide agarose gels. The method combines the high resolution fractionation of RNA by electrophoresis under denaturing conditions with the convenience of subsequent electrophoretic transfer to sheets of DEAE-membrane. This approach overcomes many of the problems associated with other procedures for recovery of nucleic acids from gels and offers the following advantages: 1) The pattern of separation of RNA achieved in the gel is preserved in the transfer to the membrane. 2) DEAE-membrane is sturdy and easy to handle when wet. 3) The membrane can be cut into very narrow sections allowing separation of RNAs migrating close together. 4) RNA can be eluted from the membrane in small volumes, thus minimizing dilution and facilitating the handling of many samples. 5) The DEAE-membrane with bound RNA can be stored for later use. 6) A permanent replicate copy of the pattern of the DEAE-bound RNA can be made by transfer of an identical RNA sample to nitrocellulose or activated paper (2, 26). Alternatively, it may be possible to bind RNA to the DEAE-membrane irreversibly by heating under vacuum as for nitrocellulose membranes.

It has recently been reported that mRNA can be translated in vitro while it is still bound to an ion-exchange cellulose support (6). This finding raises the possibility that the same reaction could be carried out with RNA bound to the DEAE-membrane support. If so, it might prove useful

for some applications. For such experiments the DEAE-membrane would probably be superior to the cellulose support because it would remain intact. We believe, however, that it is generally more advantageous to be able to recover the RNA from the support, analyze a portion of it by in vitro translation, and store the remainder frozen for later use.

We have used methylmercuric hydroxide as the denaturant during the fractionation of RNA because mild chemical conditions were already known for removing this modifying group (11). A potential variation of the methodology described here would be its adaption to use with either glyoxal (32) or formaldehyde (33, 34), denaturing agents which are less toxic than methylmercuric hydroxide. However, the limitation of these chemicals lies in the problem of reversing the modification in order to restore the biological activity of the RNA. The conditions which are known for the reversal of glyoxal modification (26, 32), as well as the conditions which have been reported for reversal of formaldehyde-induced crosslinks between proteins or between proteins and DNA (35), are all harsh treatments unsuitable for the recovery of biologically active RNA. Although our preliminary attempts to reverse the formaldehyde modification under mild conditions were unsuccessful, this is a problem which warrants further investigation.

Conceptually, the method is not restricted to the fractionation of nucleic acids under fully denaturing conditions. For some studies it would be desirable to use partially-denaturing gels, such as those containing urea or formamide, or native gels. The principles of the method are applicable to DNA, protein, and nucleic acid-protein complexes. It should therefore be possible to adapt the experimental conditions to these molecules. Thus this approach should be useful in a wide variety of investigations.

The method described here is applicable to virtually all RNAs from any biological system. In order to exemplify its use, we have isolated Xenopus albumin mRNA because its in vitro translation product could be readily recognized and the identity of complementary cDNA clones could be independently confirmed. We are currently isolating and characterizing the mRNAs for Xenopus fibrinogen subunits, a set of proteins synthesized from a family of genes under coordinate regulation by steroid hormones (19).

ABBREVIATIONS

CH_3HgOH, methylmercuric hydroxide; DEAE, diethylaminoethyl; E Buffer, .05M boric acid, .005M $Na_2B_4O_7 \cdot 10H_2O$, .01M sodium sulfate, .001M Na_2EDTA, pH 8.19; SDS, sodium dodecyl sulfate; SSC, 0.15M NaCl, 0.015M Na-citrate

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