A new blotting medium for the simple isolation and identification of highly resolved messenger RNA¹

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ABSTRACT

A simple method has been developed that allows the rapid isolation and identification of highly resolved mRNA molecules. RNA species are separated by gel electrophoresis and then blotted on to a paper sheet to which polyuridylic acid has been covalently bound. This mRNA affinity paper ("mAP") specifically binds, in a reversible manner, polyA⁺ containing molecules. A replica picture of the agarose gel is thus obtained on the mAP, from which bound mRNA molecules can be eluted by heating in water. In addition to their simple isolation individual mRNA species, whilst still bound to mAP, can be identified by both "in-situ" hybridization and translation.

INTRODUCTION

Although the preparative recovery of highly resolved mRNA fractions is of great importance especially in cDNA cloning experiments, there is, to date, no single easy and reliable method for their isolation. Gradient centrifugation has been used but this does not resolve complex mRNA populations very well - at the best 5-8 fold enrichment is obtained (1, 2). The method of choice is either agarose or polyacrylamide gel electrophoresis which allows for high analytical resolution. Although this method has been used for the isolation of biologically active mRNA, mRNA elution is both cumbersome and tedious involving the slicing of the agarose gel, melting of slices, phenol extraction and ethanol precipitation (3, 4). More recently, methods have been published in which RNA species first resolved by gel electrophoresis are transferred to a carrier support such as DEAE or ECTEOLA paper which binds the RNA by electrostatic interactions (5, 6). The subsequent isolation of RNA requires the use of high ionic strength solutions thus necessitating the removal of large amounts of salt from the isolated RNA species. Moreover these methods are not specific for mRNA.

Here we present the simple isolation of mRNA from a gel electrophoresis

matrix based on the reversible binding of polyA⁺ RNA to polyuridylic acid immobilized by covalent linkage onto a paper carrier support. RNA is first resolved by agarose or acrylamide gel electrophoresis and then transferred either by electrotransfer or blotting to the polyU paper (mAP) to which only polyA⁺ RNA binds by hydrogen bond formation. PolyA⁺ RNA whilst still bound to the mAP can either be identified by hybridization with complementary DNA probes or characterized by *in vitro* translation. The bound polyA⁺ RNA can subsequently be eluted simply by heating slices of mAP in distilled water. The highly resolved mRNA is thus obtained intact, in distilled water and free from contaminating salts or remnants of agarose or acrylamide. Moreover mAP is specific for mRNA and DNA or ribosomal RNA do not bind.

As blotting procedures have been traditionally named according to geographical directions (7-9), we propose for this new technique the name "Middle Eastern" Blotting.

METHODS

Isolation of rabbit 9S globin mRNA.

Purified rabbit reticulocyte ribosomes were isolated and extracted with phenol-chloroform as previously described (10, 11). The polysomal RNA was loaded onto a polyU-Sepharose column equilibrated in 500 mM NaCl, 10 mM Tris-HCl pH 7.6, 0.1% SDS and the polyA⁺ RNA (predominantly 9S globin mRNA) eluted with water at 50°C. The RNA was precipitated by adding 0.1 volumes of 2M sodium acetate pH 5.5 and 2.5 volumes absolute ethanol. In some cases pure rabbit 9S globin mRNA from Bethesda Research Laboratories was used.

3' End Labelling of RNA.

RNA was radioactively labelled by the 3' addition of [5'-³²P] pCp (2-3 x 10⁶ Ci/mol) using T4 RNA ligase (12). Typical ligation reactions contained in a final volume of 50 µl : 3 µl T4 RNA ligase (at 3,700 units/ml in 50% glycerol, 20 mM Tris/HCl pH 7.5, 5 mM dithiothreitol and 50 µM ATP), 25 µl ligase buffer (100 mM Hepes pH 7.6, 15 mM MgCl₂, 6.6 mM dithiothreitol and 20% v/v dimethylsulfoxide), 5 µM ATP, 100 µM TTP, 100 µCi [5'-³²P] pCp (lyophilized) and 2 µg RNA. The ligation reactions were incubated at 4°C overnight. Ligation was terminated by the addition of 5 volumes of 100 mM NaCl, 100 mM Tris/HCl pH 8.0, 1 mM EDTA and then extracted twice with phenol/chloroform. Unincorporated [5'-³²P] pCp was removed from the end labelled RNA by chromatography on a 6 ml Sephadex

G-100 column.

Preparation of PolyU paper (mAP)

Polyuridylic acid was covalently bound to diazo-thio phenyl paper (DTP or activated paper) (13). The activated paper immediately following its diazotization was washed extensively in 50 mM sodium acetate, pH 6.0. Any contaminating small amine molecules were removed from the poly-U solution. Ten millilitres (ml) of the poly-U solution at a final concentration of 2 mg/ml in 50 mM sodium acetate pH 5.5 buffer were added directly to a 20 x 15 cm sheet of the activated paper. The sheet was sealed in a plastic bag and incubated at 40°C for 48 hours.

In order to inactivate any excess diazonium groups and to saturate non-specific binding sites for nucleic acid, the poly-U paper (mAP) was washed for 5 minutes in 50 ml of wash buffer which contains the following: 50 mM NaCl, 100 mM Tris-HCl pH 7.6, 1% glycine, 0.02% polyvinylpyrrolidone (Sigma, average M.W. 360,000) 0.02% Ficoll (Pharmacia, average M.W. 400,000 daltons) and 100 microgram (µg)/ml commercially available transfer RNA. The mAP was then incubated at 40°C with 4 changes of the above wash buffer (50 ml each wash) for 24 hours, washed twice (60 minutes each wash) at 20°C with 500 ml of 500 mM NaCl, 10 mM Tris-HCl pH 7.6 followed by 2 washes for 60 minutes with 500 ml of distilled water at 20°C and then incubated in boiling distilled water for 3 minutes, washed twice for 10 minutes in 50 ml distilled water at 20°C, dried and stored at -20°C.

Gel Electrophoresis

a) Non-Denaturing-RNA samples were run in 1.5% agarose gels with 10 mM sodium-phosphate buffer pH 7.0. Electrophoresis was performed at 80 mA with buffer recirculation. RNA samples were heated to 90°C for 2 minutes prior to loading.

b) Denaturing for subsequent transfer to mAP-RNA samples may be run under denaturing conditions providing that "non-invasive" techniques are used. These include agarose gels using the urea/citrate system (14) and acrylamide gels with the urea/Tris/borate, EDTA system (15).

For analytical purposes (not for subsequent transfer to mAP) the glyoxal/DMSO technique was used (16).

Blotting

a) "Southern" blotting - following electrophoresis the gel was equilibrated in 500 mM NaCl, 20 mM Tris/HCl pH 7.4, and blotted onto mAP using the above solution as described by Southern (7).

b) Electrotransfer - following electrophoresis the gel was

Figure 1. Binding of 9S globin mRNA to mAP

9S globin mRNA was purified, radioactively labelled at the 3' end with [5'-³²P] pCp and analysed in 4 adjacent slots on a 1.5% agarose gel in 10 mM sodium phosphate buffer pH 7.0 as described in Methods. Following electrophoresis the gel was equilibrated in 500 mM NaCl, 10 mM Tris/HCl pH 7.6 and blotted onto 2 sheets of mAP. The gel after blotting (A) the 1st (B) and 2nd (C) mAP sheets were dried and autoradiographed.

equilibrated in 0.12M Na phosphate pH 6.9 and electrotransferred in the same buffer using the Trans Blot Biorad System, with a current of 1.2 Amps for 3 hours.

Elution of mRNA bound to mAP

mRNA was stably bound to mAP in salt concentrations of at least 0.1 M. The bound mRNA was eluted by immersing mAP in water and heating to 70°C for 2 minutes.

cDNA preparation

Radioactive DNA complementary to rabbit globin mRNA was prepared by reverse transcription (17). Unincorporated radioactive nucleotide was removed from the cDNA by passage through a 5 ml Sephadex G-50 column.

Reticulocyte lysate in vitro translation

This was performed using micrococcal nuclease treated lysates (18).

RESULTS

Binding of mRNA to mAP

The purification of individual mRNA species presents a serious technical problem. We reasoned that RNA species resolved by gel electrophoresis could be transferred to a solid carrier support from which they could be subsequently eluted. The proposed solid carrier should then meet the following criteria: a) Specificity of binding for polyA⁺RNA (mRNA), b) Non covalent and preferably non-electrostatic reversible binding and c) Ease of isolation of the bound mRNA.

As many species of mRNA contain a 3' polyadenylic acid tail that can

Figure 2. Binding of 9S globin mRNA to mAP and "mock" paper

9S globin mRNA end labelled with $[5'-^{32}\text{P}]\text{pCp}$ was electrophoresed on a 1.5% agarose gel and blotted onto mAP (A') or "mock" paper prepared without the addition of polyuridylic acid (B'). A and B are autoradiographs of the gels after blotting. Other details were as in Figure 1.

form hydrogen bonds with polyuridylic acid, it seemed possible that the covalent linkage of polyuridylic acid to paper may provide a suitable carrier support. In order to test the feasibility of such an approach, 9S globin mRNA was first separated by agarose gel electrophoresis and subsequently blotted on to polyU paper (mAP). Indeed, the mRNA was retained by mAP (Figure 1).

Specificity for mAP

In order to ascertain that the interaction of polyA^+ RNA with mAP was due to the presence of polyuridylic acid on the carrier support, a similar blot of 9S globin mRNA was performed onto "mock" paper prepared without the addition of polyU. Figure 2 shows that whilst almost all the mRNA bound to the mAP, little if any was retained by the "mock" paper.

Isolation from the mAP

As the binding of PolyA^+ RNA to mAP is dependent on hydrogen bond formation, the PolyA^+ RNA once bound can be eluted by heating in water. Discrete mRNA fractions can be isolated by cutting the mAP into strips and eluting mRNA from any desired strip. Figure 3 shows intact 9S globin mRNA isolated in such a manner.

Blotting of PolyA^+ RNA to the mAP can be achieved either by passive diffusion (as in the "Southern" blot) using buffered 0.5 M NaCl or by electrophoretic transfer in 0.125M sodium phosphate buffer at pH 6.9. The polyA^+ RNA is stably bound to the mAP under salt concentrations higher than 0.125M. A brief wash of the mAP with bound PolyA^+ RNA in distilled water at 20°C or lower will release 20-30% RNA whilst heating in distilled water

Figure 3. Analysis of 9S globin mRNA released from mAP

End labelled 9S globin mRNA was electrophoresed on a 1.5% agarose gel and blotted onto mAP as in Figure 1. The bound mRNA was released from mAP by heating in distilled water to 70°C for 2 minutes and the isolated RNA analysed on a glyoxal-agarose gel, which was dried and autoradiographed (Slot A), Slot B - 9S globin mRNA prior to isolation on mAP.

to 75°C for 2 minutes releases 95% bound RNA.

The mRNA released a) is intact (Figure 3)

b) is in water without contaminating substances and can thus be directly translated or reverse transcribed.

or c) can be concentrated by either lyophilization or ethanol precipitation.

Length of Polyadenylic Acid Required for Binding to mAP

In order to ascertain the length of polyadenylic acid required for binding to mAP, polyadenylic acid was radioactively labelled at its 3' end with T4 RNA ligase and [5'-³²P]pCp, partially hydrolysed in formamide, resolved by gel electrophoresis and electrotransferred to a "sandwich" comprising mAP placed adjacent to a sheet of DEAE paper. Whilst polyadenylic acid chains of less than approximately 10 residues passed through the mAP straight onto the DEAE sheet, all larger PolyA molecules were almost quantitatively bound by the mAP (Figure 4).

Specificity of Binding for PolyA⁺ RNA

Radioactively labelled DNA markers, 5S ribosomal RNA and partially

Figure 4. Length of Polyadenylic acid required for binding to mAP

Polyadenylic acid was end labelled at its 3' end with T4 RNA Ligase and [5'- 32 P]pCp and partially hydrolysed by boiling for 30 minutes in deionized formamide. Non-hydrolysed (A) or partially hydrolysed (B) polyadenylic acid was electrophoresed on a 20% acylamide gel in 7M urea and electrotransferred to a sheet of mAP placed adjacent to DEAE paper as in Methods. Following electrotransfer the mAP and DEAE paper were dried and autoradiographed. The numbers 5 and 20 represent migration of 5 and 20 nucleotide length chains, respectively.

degraded 9S globin mRNA were electrophoretically resolved on a 5% acrylamide gels in the presence of 7M urea, and then electrotransferred to a "sandwich" comprising mAP placed adjacent to a sheet of DEAE paper. Whilst both the labelled DNA markers and 5S ribosomal RNA passed directly through the mAP and were bound by the DEAE paper only the 9S globin mRNA was retained by the mAP (Figure 5). Almost no binding of either the DNA markers or 5S ribosomal RNA to the mAP was observed.

Hybridization of mRNA Bound to mAP

Samples of 9S globin mRNA, and polyA⁺ RNA extracted from HeLa cells were electrophoretically resolved on agarose gels and electrotransferred to mAP. The mAP was probed with radioactively labelled cDNA prepared by the reverse transcription of rabbit 9S globin mRNA. Hybridization was only observed in the lane containing the 9S globin mRNA (Figure 6A). It should be noted that the RNA samples were not "baked" onto the mAP, but were simply bound by hydrogen bonding.

The sensitivity of hybridization to mRNA bound on mAP was assessed by titrating 9S globin mRNA using a dot hybridization assay. Less than 16 pg

Figure 5. Specificity of mAP for polyA⁺ RNA

5S rRNA (Slot A), partially degraded 9S globin mRNA (Slot B), end labelled at their 3' end with T4 RNA ligase and [5'³²P]pCp, and a labelled restriction digest of pBR322 (Slot C), were electrophoresed on a 5% acrylamide gel in 7M urea. The resolved samples were electrotransferred to a sheet of mAP placed adjacent to DEAE paper. Following transfer the sheets were dried and autoradiographed.

of mRNA could be detected in an overnight exposure using this method (data not shown).

Translation of mRNA bound to mAP

The translatability of mRNA bound to the mAP was investigated. Figure 7A shows that mRNA released from mAP is capable of directing in vitro translation. A linear relationship between input mRNA and protein synthesis was observed.

Interestingly mRNA still bound to mAP was able to direct protein synthesis (Fig. 7B). Part of the proteins synthesized were released into the translation solution and part remained on the paper. Protein synthesis directed by mRNA bound to mAP continued for up to 60 minutes.

DISCUSSION

We have shown that highly resolved mRNA species can be blotted onto

Figure 6. Hybridization of mRNA bound to mAP

Rabbit 9S globin mRNA (1 μ g, Slot A) and polyA⁺ RNA extracted from HeLa cells (8 μ g Slot B) were run on a 1.5% agarose gel in Tris/Acetate/EDTA buffer at pH 7.0, and electrotransferred to mAP as described in Methods. The mAP was washed for 60 minutes in 4 x SSC (1 x SSC is 0.15M NaCl, 0.015M Na citrate, pH 7.0), incubated overnight with radioactive cDNA (10⁶ cpm/ml) prepared against 9S globin mRNA, washed 4 x 30 minutes in 1 x SSC and then dried and autoradiographed.

and reversibly bound by paper to which polyuridylic acid has been covalently bound (mAP). Individual mRNA species may then be eluted in distilled water. This allows for the simple isolation of highly resolved polyA⁺ RNA in water free of salts and residual agarose or acrylamide contaminants. Moreover, using this technique there is no need to purify polyA⁺ RNA prior to gel electrophoresis - the blot procedure itself selects only polyA⁺ RNA molecules. We have also demonstrated that mAP bound mRNA can be characterized directly either with appropriate complementary probes or translated in in vitro translation systems thus simplifying the identification of relevant mRNA species for subsequent isolation.

In this paper we have used rabbit 9S globin mRNA as a model polyA⁺ RNA. However, polyA⁺ RNA extracted from Ehrlich Ascites tumor cells and polyA⁺ containing RNA from rat liver nuclei have also been successfully transferred to mAP and it is reasonable to expect that this method will be applicable for any polyA⁺ containing molecule.

Denaturing conditions can be used for the electrophoresis of RNA samples prior to transfer to the mAP as long as the RNA molecules are not modified covalently. Thus, whilst glyoxal denaturation should be avoided,

Figure 7. Translation of mRNA bound by mAP

Different amounts of rabbit 9S globin mRNA in 0.5M NaCl, 10 mM Tris/HCl pH 7.6 were spotted onto small pieces of mAP, incubated at room temperature for 30 minutes and then briefly washed in cold distilled water. The mAP-bound mRNA was eluted by heating in water at 70°C for 2 minutes and the isolated mRNA translated in a micrococcal nuclease treated reticulocyte lysate system (Fig.7A). Figure 7B shows protein synthesis directed by 9S globin mRNA whilst still bound on the mAP. Open circles represent protein synthesis without the addition of mRNA.

the use of urea as a denaturant for example is compatible with this technique. For initial purification of mRNA species it may be sufficient to simply electrophorese RNA under non-denaturing conditions.

mRNA whilst still bound to mAP may be analysed by complementary hybridization as well as by *in vitro* translation. Hybridization is performed with the polyA⁺ RNA hydrogen-bonded onto the mAP. Thus once a certain mRNA species has been positively identified by hybridization it may then be eluted by heating in water. Hybridization sensitivity using mAP is comparable to and possibly even better than other published procedures (8) in which the RNA is irreversibly baked onto nitrocellulose filters and it is both more sensitive and simpler than hybridizations using the ECTEOLA paper technique (6). Alternatively the mRNA of interest may be identified by the translational products of an *in vitro* translation. As we have shown (Fig. 3) mRNA released from the mAP by heating in distilled water is undegraded and intact so that the translational efficiency will be accordingly high.

The procedure we have described here also opens up the possibility of using highly purified radioactively labelled mRNA probes to directly screen recombinant DNA libraries for relevant clones. It seems feasible that mRNA

species of interest may be purified with mAP, using a 2-dimensional gel system (employing, for example, different buffers in each dimension), end-labelled with $[5^{32}\text{P}]\text{pCp}$ and T4RNA ligase and then used as probes to screen a cDNA or genomic library. Such an approach should simplify the positive identification of recombinants.

The potential of mAP as an aid in cloning experiments especially with regard to low abundant mRNAs seems to be great. The technique should thus simplify current cloning procedures.

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1 - Patent Pending.

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