Blotting of RNA onto ion exchange paper allowing subsequent characterization by in situ translation in addition to blot hybridization

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ABSTRACT

We present the preparation of an ion exchange paper which can be used as a solid carrier in the transfer of RNA from gels. In addition to detection by blot hybridization to specific probes, transferred RNA can be characterized by cell-free translation in situ. Preparation of the paper is simple, inexpensive and reproducible. Examples of applications are shown and possible other applications are discussed.

INTRODUCTION

Transfer of DNA (1) and more recently of RNA (2, 3) from gels onto a solid carrier has become an important tool in molecular biology over the past few years. In these so-called blotting procedures, subsequent characterization of the transferred nucleic acids, is limited to detection by hybridization to radioactive probes. The ease with which individual RNA species are separated on and recovered from gels by blotting, prompted us to seek a modification of this procedure that would allow characterization of transferred RNA by in vivo translation in addition to detection by hybridization. Our strategy was to develop a solid carrier that would bind RNA in a non-covalent fashion and such that the RNA could be eluted in a volatile buffer from which it could be recovered for in vitro translation by lyophilization. A volatile elution buffer was chosen because ethanol precipitation of RNA from any other buffer system would require the addition of relatively large amounts of carrier material which might interfere with subsequent analyses.

An obvious choice for non-covalent binding being electrostatic interactions, we tested a number of ion exchange papers for their binding characteristics by chromatography of RNA using two different buffer systems. Criteria for suitable binding were: low mobility of RNA at moderate ionic strength and neutral pH (100 mM KAc-HAc pH 7.4) and high mobility at high ionic strength and elevated pH (30 % triethylamine carbonate, TEC, pH 9.0). It soon became
apparent that commercially available ion exchange papers (DEAE cellulose and Servacel WB-2) and ECTEOLA paper synthesized according to published procedures (4) all bound RNA, larger than 10 S, too strongly; no buffer system could be found in which RNA could be eluted intact. We therefore set out to modify the synthesis of ECTEOLA paper such that RNA binding was sufficiently weakened. A good compromise between the two binding criteria was found in the procedure presented here. RNA was quantitatively bound to the paper in transfer from agarose gels and after elution from the paper and lyophilization the RNA could be accurately translated in a cell-free system (not shown). An even more practical procedure was found in translating RNA while still bound to the paper, by immersing and incubating an RNA containing section of the blot in translation mixture.

In this report we will refer to in vitro translation of ECTEOLA paper-bound RNA as in situ translation. During translation, RNA remained bound to the paper and, in addition, cell-free translational products were immobilized at their site of synthesis. This latter aspect of the in situ translation allowed us to develop a two-dimensional procedure which involves in situ translation of an entire strip of the ECTEOLA blot and subsequent polyacrylamide gel electrophoresis of the translation products. To be able to correlate in situ translational products with specific RNA species, detected by hybridization to specific probes, we needed to covalently couple the RNA to the paper, since the electrostatic interactions were too weak to bind RNA under conditions generally used for hybridization. A satisfactory procedure was found in the carbodiimide mediated phosphodiester bond formation between terminal phosphates of the RNA and hydroxyl groups of the paper, according to the method described by Gilham (5).

The problem of 2'-3' cyclic phosphate formation in this reaction was overcome by generating 5' phosphates by limited fragmentation of paper-bound RNA with P1 nuclease.

We here present examples of the ECTEOLA blotting procedure and discuss possible applications.

MATERIALS AND METHODS

Preparation of ECTEOLA paper

The reaction protocol described here is based on the procedure of Peterson and Sober (4). Several adaptations however were needed to make it suitable for the specific purpose of blotting of large RNA molecules. Since blotting requires sheets of the solid carrier rather than a suspension (for which the published
procedure is described) a number of problems connected with this aspect had to be worked out. Firstly, a number of different kinds of paper was tested since the source of the cellulose (wood or cotton) strongly influences the reactivity. Of the papers tested, Whatman 1 MM gave the best results. Secondly, when using paper sheets, the reaction mixture does not remain homogeneous because it can not be stirred vigorously as is the case with cellulose powder. This problem was overcome by altering the sequence in which the reactants were added.

Finally, a large number of different reactant ratios were tested to yield a paper with the desired binding characteristics. Binding criteria were: immobilization of (ribosomal) RNA in 100 mM KAc-HAc pH 7.4 and release in 30 % TEC pH 9.0. The resulting protocol is as follows: a sheet of Whatman 1 MM paper was immersed in 10 M NaOH in a Pyrex oven dish at 0° C for 30 minutes. Excess NaOH was blotted by firmly pressing the sheet between two layers of Whatman 3 MM paper. Pressure was applied with a rolling device, commonly used in the drying of photographic prints. The sheet was then immersed for 15 minutes at room temperature (fume hood) in a reaction mixture which typically consisted of 8.1 g triethanolamine (Merck, Darmstadt, West Germany) and 22.5 ml epichlorohydrin (Merck). Excess reaction mixture was blotted as described before for NaOH and the sheet was placed back in the Pyrex dish with 10 M NaOH. The dish was transferred to a waterbath and kept at 85-90° C for 2 hours. Care was taken that the sheet remained fully submerged in NaOH during the reaction by placing glass weights along the edges. During the reaction the sheet should not turn yellowish which occasionally did happen when excess reaction mixture had not been blotted off sufficiently. Yellowish ECTEOLA paper will bind RNA too strongly. Reaction was stopped by placing the ECTEOLA sheet in ice cold 3 M KAc-HAc pH 7.4, followed by three successive washings in 1 M HAc and 1 M KOH. Finally, the paper was washed until neutral with water and air dried after washing in absolute ethanol.

ECTEOLA blotting
Moloney murine leukemia virus (M-MuLV) 70S RNA was isolated from virions (harvested at 2 hours or 12 hours intervals) by Proteinase K digestion and sucrose gradient centrifugation. Cytoplasmic RNA from virus-infected tissue culture cells was isolated by magnesium-precipitation according to Palmiter (6), followed by oligo dt-cellulose (Collaborative Research, T-3 grade) chromatography. RNA was separated on 2 mm thick vertical 1 % acid urea agarose gels according to Rosen et al. (7). After electrophoresis, RNA was blotted onto ECTEOLA paper following the procedure of Southern (1) using 100 mM KAc-HAc pH 7.4 as transfer buffer. In the experiments shown here transfer was for
16 hours at 4°C, although transfer is probably complete in a much shorter time. ECTEOLA blots were washed in transfer buffer and stored until further use under 1 volume of transfer buffer and 2 volumes of ethanol at -20°C.

**Blot-hybridization**

For covalent binding of RNA to ECTEOLA paper the blot was first treated with PI nuclease to generate 5'-phosphates. Blots were washed in PI buffer (10 mM NaAc-HAc pH 5.3, 0.4 mM ZnSO₄, 4 mg/ml bovine serum albumin) and incubated for 1 hour at 37°C in 15 μl/cm² of PI buffer containing 10⁻³ units/ml PI nuclease (Boehringer, Mannheim) and 33 μg/ml rabbit tRNA. Subsequently, the blot was washed in 20 mM sodium 2-(N-morpholino) ethanesulfonate (MES) pH 6.0, 5 mM EDTA followed by extensive washing in water and complete drying in vacuo at room temperature. Then the blot was gently agitated for 24 hours at room temperature in excess carbodiimide reaction mixture, typically prepared by dissolving 150 mg N-cyclohexyl-N'-B-(4-methylmorpholinum)ethyl carbodiimide p-toluensulfonate (Merck) in 0.3 ml 0.2 M MES pH 6.0 and 2.4 ml water. After the reaction the blot was briefly washed in, successively, dilute ammonia pH 9.0; 5 mM HEPES pH 7.5; and pre-hybridization buffer. Hybridization to ³²P nick translated probes was for 16 hours at 41°C, essentially as described by Adkins et al. (8).

Hybridization to M-MuLV specific cDNA (prepared according to Van der Putten et al. (9)) was for 4.5 hours at 68°C in 10 mM TES pH 7.4, 1 mM EDTA, 1 M NaCl, 0.2 % SDS, 2 μg/ml polyvinylchloride, 1 mg/ml denatured calf thymus DNA, 10 μg/ml poly A, 5 x Denhardt solution, 20 mM phosphate, followed by successive washings in 50 % formamide, 5 x SSC, 1 % SDS and 2 x SSC, 1 % SDS at 41°C, and 0.1 x SSC, 0.1 % SDS at 50°C.

**Blot-translation and SDS-PAGE**

Strips or sections of ECTEOLA paper were washed in 100 mM KAc-HAc pH 7.4, blotted between Whatman 3 MM paper and saturated (5 minutes at 0°C) with excess complete translation mixture, only lacking radioactive amino acid(s). ECTEOLA was again firmly pressed between two layers of 3 MM paper and incubated in 33 μl/cm² of complete translation mixture for 2 hours at 30°C. Small paper sections were incubated in 0.5 ml Eppendorf vials, entire strips in sealed plastic bags. In situ translational products were released from the paper in electrophoresis sample buffer (10 M urea, 62.5 mM Tris-HCl pH 6.8, 3 % SDS, 5 % 2-mercaptoethanol) and analyzed on 10 % polyacrylamide slab gels according to the discontinuous system of Laemmli (10).

For SDS-PAGE of entire strips, the strips were sealed in low melting agarose
(Sea-plaque) in the gel slot after washing in 62.5 mM Tris-HCl pH 6.8, 5 % 2-mercaptoethanol. The cell-free translation system used was the mRNA dependant reticulocyte cell-free system prepared essentially as described by Pelham and Jackson (11), except that white blood cells were removed by chromatography over SP-Sepharose after Perucho et al. (12).

Reticulocyte lysates were treated with micrococcal nuclease in the presence of 2 mM DTT, 10 µg/ml hemin, 50 µg/ml creatine kinase and stored at -70° C. Typical translation mixtures (final volume 200 µl) contained 120 µl of reticulocyte lysate and (added) final concentrations of 100 µg/ml creatine kinase, 10 mM creatine phosphate, 0.3 mM spermidine-HCl, 0.1 mM of each unlabeled amino acid (always including glutamine), 50 µg/ml rabbit tRNA, 1 mM MgAc₂, 100 mM KAc, 1 mM ATP, 0.2 mM GTP in addition to radioactive amino acid(s).

RESULTS

In situ translation

Having modified the synthesis of ECTEOLA paper to yield a solid carrier that retains RNA in blotting from agarose gels in 100 mM KAc-HAc pH 7.4 and releases it in 30 % TEC pH 9.0, we found that in situ translation could be performed with the RNA still bound to the paper (in situ). Immersing and incubating the paper-bound RNA in translation mixture directly after transfer from the gel, resulted however in many artifactual translation products; mostly premature terminations. After many unsuccessful attempts to improve the translation accuracy by altering the concentration of individual constituents of the translation mixture, we found that translation could be performed with great fidelity by first soaking the RNA containing paper with complete translation mixture in which only the radioactive amino acid had been omitted. It seems that binding sites on the paper need to be saturated with certain factors to prevent depletion of the translation mixture.

A comparison of translation products of increasing concentrations of Moloney murine leukemia virus (M-MuLV) 70 S virion RNA translated either in solution (lanes 1-4) or in situ on ECTEOLA paper (lanes 5-8) is shown in Figure 1. The autoradiogram of the analytical SDS gel shows that with a small reduction in the amount of high molecular weight polypeptides, the in situ translation yields a set of products which is virtually indistinguishable from the set synthesized in solution. Later we found that the small reduction in high molecular weight proteins is due to retention on the ion exchange paper rather than to the quality of the translation itself. The problem can easily be overcome by including the paper piece in the slot on the analytical SDS gel (not
shown). Also we found that spotting the RNA on ECTEOLA paper in a denaturing buffer (the same buffer as used for RNA gel electrophoresis) rather than in water, slightly increases the in situ translation efficiency. Thus, in situ translation of RNA bound to ECTEOLA paper is highly accurate and makes elution of RNA from the paper for this purpose superfluous.

**Blot hybridization**

In order to show detection of specific RNA species by hybridization, purified M-MuLV 35S virion RNA, poly A selected cytoplasmic RNA from Moloney sarcoma virus (MSV) transformed M-MuLV infected cells, and $^{32}$P labeled RNA from M13 infected E coli cells were electrophoresed on acid urea agarose gels (7) and blotted onto ECTEOLA paper. After limited PI nuclease digestion and carbodiimide coupling the blot was divided in two sections and autoradiographed (Fig. 2) either directly (lane 1) or after hybridization to $^{32}$P labeled M-MuLV specific cDNA (lanes 2 and 3).

Bands at 35S (M-MuLV) and at 30S (MSV) are detected specifically against a low background hybridization. The sharp marker bands in lane 1 indicate that no spreading of RNA takes place during PI nuclease digestion and carbodiimide
Fig. 2. Autoradiography of an ECTEOLA blot. RNA was separated on a 1% acid urea agarose gel, transferred to ECTEOLA paper and covalently bound by carbodiimide treatment after limited PI nuclease digestion. The blot was divided in two and autoradiographed either directly (lane 1) or after hybridization to M-MuLV specific cDNA (lanes 2 and 3). RNAs were: $^{32}$P labeled RNA from M13 infected E.coli (lane 1); sucrose gradient purified M-MuLV 35S virion RNA (isolated from virions harvested at 2 hours intervals) (lane 2); and poly A selected cytoplasmic RNA from Clone 124 MSV transformed M-MuLV infected cells (lane 3).

reaction. The sensitivity of detection is comparable to what we find (not shown) with blotting onto nitrocellulose following the procedure of Thomas (3).

Correlation of translation and hybridization profiles
To demonstrate the possibility of accurately correlating in situ translational products with specific bands in the hybridization pattern, we show an analysis of poly A selected cytoplasmic RNA from tissue culture cells and of retroviral virion RNA. RNA was separated on an acid urea agarose gel and transferred to two layers of ECTEOLA paper. The lower layer consisted of two separate (1 x 11 cm) strips which were placed on top of and in direct contact with the gel, each covering the lengthwise central section of one (2 cm wide) lane. The strips were notched along one side to allow accurate alignment of paper sections used for in situ translation with the hybridization profile of an upper layer of ECTEOLA (6 x 11 cm) which covered the two strips and the rest of the gel. This way, RNA migrating within the central 1 cm of each lane was transferred to the lower layer of ECTEOLA (the strips) whereas material migrating along the edges (0.5 cm on either side) was transferred to the upper layer. To prevent degradation of transferred RNA, the ECTEOLA layers were
stored until further use under 70% ethanol, 33 mM KAc at -20°C, immediately after transfer. After treatment with PI nuclease and carbodiimide, the upper layer of ECTEOLA was hybridized to a $^{32}$P nick translated probe and autoradiographed (Fig. 3, left panel).

The RNA in lane B had been isolated from Abelson leukemia virus (AbLV) transformed ANN cells which were productively infected with M-MuLV. Although the AbLV genome contains sequences related to M-MuLV, the probe (cloned proviral M-MuLV DNA; pMLV) does not clearly detect AbLV specific 30 S RNA. This is probably due to relatively low concentrations of this RNA species and to the nature of the probe (M-MuLV specific rather than AbLV specific, and nick...

Fig. 3. Left panel. M-MuLV 70 S virion RNA (isolated from virions harvested at 12 hour intervals) (lane A), and poly A selected cytoplasmic RNA from AbLV transformed M-MuLV infected cells (lane B) were electrophorezed on a 1% acid urea agarose gel and transferred to two layers of ECTEOLA paper as described in the text. The upper layer was hybridized to $^{32}$P nick translated cloned M-MuLV proviral DNA (pMLV). The autoradiogram is shown.

Right panel. SDS-PAGE analysis of in situ translational products. Lower layer ECTEOLA strips (see text) were lengthwise cut in half and the notched half was subdivided in 4 x 4 mm sections (spanning a single notch each). Sections indicated in the left panel were analyzed by SDS-PAGE after in situ translation.
translated cloned DNA rather than cDNA). In general we found that cDNA probes (as used in Fig. 2) gave better results than nick translated probes.

M-MuLV specific RNA is detected as discrete bands at 35S (genomic RNA, gag mRNA and gag-pol mRNA) and at 21S (env mRNA). The faint background hybridization within the lane, starting at 35S, indicates that breakdown of RNA occurred prior to or during electrophoresis.

The native M-MuLV 70S virion RNA (lane A) is a dimer of 35S genomic RNA, complexed with some minor contaminants of both viral and cellular RNA's. As is to be expected for total RNA isolated from virions harvested at 12 hours intervals, the 35S RNA is substantially fragmented. The absence of hybridization in the center of both lanes A and B indicates that RNA from this part of each lane was quantitatively retained by the lower layer ECTEOLA strips in the transfer procedure.

These lower layer strips were cut in half lengthwise and translated in situ as described above for spotted RNA. The smooth edge half was translated entirely and the further analysis of it, which is shown in Figure 4, will be discussed later. The notched half was subdivided as indicated in Figure 3 (left panel) and individual sections, each spanning a single notch, were translated. Product analysis by SDS-PAGE of sections surrounding the position of 35S is shown in Figure 3 (right panel). Clearly the onset of synthesis of 35S RNA encoded Pr65\(^{\text{gap}}\) coincides with the position of 35S RNA in the hybridization profile. Thus the ECTEOLA blotting procedure allows translational products to be accurately correlated with specific RNA species.

A two-dimensional version of the blotting procedure is shown in Figure 4, where translational products of the entire smooth edged half of the lower layer strip of lane B in Figure 3 (left panel) were analyzed by SDS-PAGE. After translation incubation in a sealed plastic bag, the strip was washed and placed horizontally in a wide slot in the stacking gel. To prevent horizontal spreading of protein by direct contact of the strip with electrophoresis sample buffer, the strip was first sealed in the slot with low melting agarose which, after solidifying, was overlaid with sample buffer and electrophoresis buffer. In separate small slots on either side of the wide slot, sections of in situ translated ECTEOLA paper were placed, onto which M-MuLV virion RNA (A) and poly A RNA from AbLV/M-MuLV infected ANN cells (B) had been spotted. The autoradiogram, consisting of sections with different exposure times, shows that the majority of translational activity migrates at the bottom of the RNA gel (15-20 S). The fact that the translational products of RNA in this size range appear as discrete spots, indicates that these RNAs migrated as discrete
Fig. 4. SDS-PAGE analysis of in situ translational products of an entire top to bottom strip of the ECTEOLA blot of which the hybridization profile was shown in Figure 3, (left panel, lane B), and which is again shown horizontally at the top of this figure. The smooth edged strip was cut from the lower layer notched strip as indicated in this figure and was prepared for in situ translation and subsequent SDS-PAGE analysis as described in the text. The RNA preparations which were analyzed by gel electrophoresis in the left panel of Figure 3 (A and B) were also spotted on 4 x 4 mm ECTEOLA paper sections and their in situ translational products were run alongside those of the horizontal strip in this figure. The autoradiogram was composed of different exposures of gel sections which are indicated at the bottom. Exposure was for 6 hours on slow X-Ray film (section 1) and for 24 hours (section 2) and 1 week (section 3) on fast film.
bands and that no horizontal spreading of protein occurred during in situ translation and subsequent analysis. In contrast, the products of the larger RNAs appear as horizontal bands. The set of bands starting at a discrete position in the SDS gel, which coincides with the position of 35S RNA in the hybridization profile (shown at the top), is characteristic for translational products of M-MuLV virion RNA (lane A on the left). Apparently the high molecular weight RNA is predominantly virus specific. As was already evident from the hybridization profile this RNA appears to be substantially degraded. We therefore believe that the appearance of bands instead of spots in this region of the SDS gel is due to translation of RNAs truncated at their 3' ends, rather than to horizontal spreading of protein during or after in situ translation. Thus the two-dimensional ECTEOLA blotting procedure allows rapid analysis of translational products of a large number of individual RNA species separated on gels.

DISCUSSION

We presented an RNA blotting procedure that allows subsequent characterization of transferred RNA by hybridization as well as by cell-free translation. The efficiency and accuracy of in situ translation of paper-bound RNA was shown to be at least as good as translation of RNA in solution. Since in situ translational products are released from the paper in electrophoresis sample buffer, they can be analyzed by immunoprecipitation (not shown) following the procedure of Dougherty and Hiebert (13). Although we have only shown examples of transfer of RNA from acid urea agarose gels, the retention of RNA by ECTEOLA paper being based on electrostatic interactions, transfer can probably be conducted from other gel systems as well. An interesting aspect of the binding properties of ECTEOLA paper is that, at neutral pH and moderate ionic strength, in addition to RNA, also protein is bound. This aspect made it possible to analyze entire strips of ECTEOLA blots in a two-dimensional fashion. Analyses of this kind are particularly suitable for the study of translational products of RNA species which migrate very close together in the RNA gel.

M-MuLV 35S RNA is an example of an RNA which in fact may consist of several closely related species. The viral genome contains the genes gag, pol and env (in this 5'-3' order). Amino acid sequences related to the gag and pol genes occur in various overlapping precursor proteins, such as Pr65\textsuperscript{gag}, Pr75\textsuperscript{gag} and Pr180\textsuperscript{gag-pol}. The DNA sequence of this virus (14) reveals that for the synthesis of at least one of the gag-gene encoded polyproteins (Pr75\textsuperscript{gag}) a (small)
splice is required, and thus one or more 35S mRNAs may exist which are slightly different from genomic RNA. A similar conclusion can be drawn from a comparison of the translational efficiency (not shown) of M-MuLV virion RNA and of mRNA from M-MuLV infected cells. The relatively low translation efficiency of virion RNA for both Pr65\textsuperscript{gag} and Pr75\textsuperscript{gag} suggests that translational activity of this RNA may in fact reside in a small amount of contaminating true mRNA(s) rather than in the genomic RNA itself. Also separate 35S mRNA species for the synthesis of gag-pol readthrough products may be generated by a small splice at the gag-pol-junction. Readthrough into the pol-gene can be enhanced by suppressor tRNAs (15) but it has not been shown that this mechanism is indeed operative \textit{in vivo}. Although small splices are unlikely to cause physical separation of these large (hypothetical) mRNA species on denaturing gels, their effect on the secondary structure may be sufficient to result in separation on semi-denaturing gels such as acid urea agarose gels. The analysis, shown in Figure 4, reveals that the synthesis of all gag- and gag-pol-related polypeptides commences at a position which coincides with the 35S band in the hybridization profile. These include Pr65\textsuperscript{gag}, Pr75\textsuperscript{gag} (arrows) and a number of polyproteins between 80-180 kD which have been shown to be premature terminations of Pr180\textsuperscript{gag-pol} (unpublished data). Thus, if indeed several M-MuLV 35S RNAs exist, they are not separated on this gel system.

The covalent coupling of RNA to ECTEOLA paper is rather laborious. When only hybridization profiles are wanted, ECTEOLA blotting is by no means advantageous over established procedures. However, when individual RNA species are to be correlated with translational products, the procedure is far more sensitive and rapid than the only alternative, which is elution of RNA from gel slices which in addition generally involves oligo dT-cellulose chromatography requiring the presence of poly A sequences. Recently we found that RNA can be irreversibly bound for hybridization purposes by simply keeping it at 50° C \textit{in vacuo} for 10 minutes after complete drying. RNA bound to ECTEOLA in this manner hybridizes specifically to radioactive probes albeit with a much lower efficiency. Thus when detection levels are not a problem, ECTEOLA blot-hybridization can be simplified considerably.

The potential of ECTEOLA paper, which apparently allows a process as complicated as protein synthesis to go on, may include the possibility of carrying out a large number of enzymatic reactions which involve nucleic acids. Conceivably, reversed transcription of ECTEOLA blotted RNA is possible and perhaps also following reactions involved in cDNA cloning may be conducted \textit{in situ}. In \textit{in situ} translation showed that also protein is bound by the paper, which
opens the possibility of blotting proteins from gels and subsequently testing their enzymatic activity.

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