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UV crosslinking of RNA to nylon membrane enhances hybridization signals

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Abstract

An improvement in the detection by nucleic acid hybridization of size-fractionated RNA immobilized to nylon-based membranes is described. Electrophoretic transfer of RNA to nylon membranes permits a quantitative determination of different RNA transcripts on the same membrane after sequential hybridization using different ^{32}P-labeled DNA probes. UV crosslinking of the RNA to the nylon membrane increased the intensity of the radioactive signals. Using the method reported here, increased signals of between 10 and 40 fold were observed, depending on the species of transcript tested. Moderately abundant as well as rare transcripts can easily be detected in as little as 5 μg total cellular RNA.

Introduction

Methods used to immobilize RNA on solid matrices and to detect the RNA using ^{32}P-labeled DNA probes have recently been reviewed (17). Among the immobilizing matrices such as diazotized cellulose (1), nitrocellulose (26), ECTEOLA cellulose (21) and DEAE cellulose (13), nitrocellulose (NC) is the most used membrane. Results obtained from protein blotting pointed to the possible use of nylon membrane in nucleic acid hybridization (9), and New England Nuclear reported a procedure (18), that varies from conditions used for NC membranes (26).

We report here a marked improvement in the use of nylon membrane for RNA hybridization. The results show that UV crosslinking (5) of the RNA to the nylon membrane increased the intensity of the radioactive signals after hybridization with ^{32}P-labeled DNA probes. We show furthermore that the same nylon membrane can be used for staining, fluorography and subsequently for sequential hybridization with different ^{32}P-labeled DNA probes.

Materials and methods

RNA extraction

Primary mouse kidney cell cultures infected with polyoma virus (16) were used 18 h after infection. To extract total RNA, cells were lysed in 2% SDS, 10 mM Tris-HCl pH 8.0, 1 mM EDTA containing 500 μg/ml proteinase K (Merck) followed by homogeneization with a syringe to shear the DNA and by incubation at 37 °C for 30 min. The homogenate was extracted twice with phenol-chloroform-isoamylalcohol (50:50:1 by vol) saturated with 50 mM Tris-HCl pH 7.5, containing 0.15 M sodium chloride and 1 mM EDTA. Cytoplasmic RNA was prepared as previously described (16) except that the cytoplasmic fraction was digested with proteinase K (500 μg/ml) before phenol extraction. RNA was twice precipitated with ethanol, digested with DNAse I (Boehringer) at 20 μg/ml for 30 min at 37 °C, reextracted with phenol and ethanol precipitated. The final RNA pellets were resuspended in 10 mM Tris-HCl pH 7.0 at a concentration of 2 μg/μl.

[^{32}P]- or [^{3}H]-labeled total RNA were prepared from CV-1 cells or from primary mouse kidney cell cultures after a one hour pulse in 3 ml phosphate-free medium or Eagle's medium containing 50 μCi of $H_3{}^{32}PO_4$ or 50 μCi (5-^{3}H) uridine (Amersham, R.C., UK), respectively.

RNA dot-blots

RNA dot-blots were performed as described (4). [^{32}P]RNA was denatured in the presence of 16.5% formaldehyde (Merck) and 1× MOPS buffer (20 mM Morpholinopropanesulfonic acid (Fluka), 5 mM sodium acetate, 1 mM EDTA, adjusted to pH 7.0 with NaOH) at 65 °C for 15 min and mixed with an equal volume of 20× SSC (1× SSC is 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0) containing bromophenol blue. Nitrocellulose (NC; Schleicher and Schuell) and Gene Screen (GS; New England Nuclear) membranes were wetted with distilled water, incubated in 10× SSC, cushioned onto three layers of dried Whatman 3MM paper and then tightly clamped inside a manifold filtration apparatus. Thirty μl aliquots of the [^{32}P] RNA mixture were added to the wells and the solutions allowed to flow through the membranes without suction. After disassembling the manifold, the membranes as well as the three successive filter papers were removed, air dried, and processed for autoradiography, followed by liquid scintillation counting.

RNA gel electrophoresis

RNA was denatured in a mixture of 6% formaldehyde and 1× MOPS buffer for 5 min at 65 °C in the presence of 50% formamide (14), unless indicated in the text. Aliquots of 10 μl containing 5 μg RNA as well as bromophenol and xylene cyanol tracking dyes and 5% Ficoll (Pharmacia Fine Chemicals) were loaded onto horizontal 1% agarose gels (0.5 cm thick, 10 cm long) made up in 6% formaldehyde and 1× MOPS buffer (15). Electrophoresis was conducted at room temperature in the presence of 1× MOPS buffer at 120 volts for 4 h or overnight at 25 volts without recirculation of the buffer.

RNA transfer onto the nylon membrane

After electrophoresis the fractionated RNAs were electrophoretically transferred from the gel to a GS membrane in 25 mM sodium phosphate buffer (pH 6.5), using a Trans-blot apparatus (Bio-Rad), as described (2). The transfer was done at 4 °C for 2 h at 30 volts (1 A) or overnight at 7.5 volts (0.15 A). In some experiments, the RNAs were transferred overnight by the capillary blot procedure (24) using 25 mM sodium phosphate, pH 6.5, as transfer buffer (18).

After transfer of the RNAs, the damp GS membrane was treated in either one of the following ways: (1) baked for 2 h at 80 °C under vacuum; (2) baked for 2 h at 80 °C without vacuum (18) or (3), the RNA-containing side was UV irradiated (5) for 2 min with a germicide Philips TUV 15 W tube at a distance of 12 cm. The dried membrane was used immediately or stored under vacuum.

Staining and fluorography of transfers

The GS membrane was soaked under constant agitation in distilled water containing 2% SDS in order to remove the bromophenol blue and xylene cyanol, followed by extensive washing in distilled water to remove the SDS. The membrane was stained with 0.04% (w/v) methylene blue in 0.5 M sodium acetate (pH 5.2) for 15 min (15), and destained in H_2O-ethanol (3:1 by vol) until stained bands were visible on a colorless background. Photographs were taken by reflexion illumination with the membrane immersed in distilled water. For fluorography, the dried membrane was soaked or sprayed with a solution of 10% (w/v) PPO (Merck) dissolved in ethanol, air dried, and exposed to Kodak XAR-5 film at −70 °C. Before processing for hybridization, the membrane was washed extensively (2 h) with ethanol in order to remove any traces of PPO, washed briefly in distilled water and air dried.

Hybridization

The prehybridization solution was freshly prepared as described (18) and contained 50% twice recrystalized and deionized formamide (Fluka), 1 M sodium chloride, 0.2% polyvinyl-pyrrolidone (mol.wt. 25000–30000; Merck), 0.2% BSA (Sigma), 0.2% ficoll (mol.wt. 400000; Pharmacia), 50 mM Tris-HCl pH 7.5, 0.1% sodium pyrophosphate, 1% SDS, 10% dextran sulfate (mol.wt. 500000; Pharmacia). The solution was slowly and

thoroughly mixed at 60 °C and filtered while hot through a 0.45 μm HA-Millipore. Hundred μg/ml of sonicated salmom sperm DNA which had been heat denatured at 75 °C for 15 min in 85% formamide were then added. The GS membrane was prehybridized for 16 h at 42 °C in a sealed bag with 0.15 ml solution per cm^2 of membrane under slow constant rocking. Aliquots of nick-translated ^{32}P-probes were heat denatured at 75 °C for 15 min in a volume of prehybridization solution (without sodium chloride, dextran sulfate and carrier DNA) equal to one fifth of that used for prehybridization, and the solution added to the bag containing the GS membrane. Final concentration of the probe was usually used at $1-1.5\times10^6$ cpm/ml. After hybridization for 24 h at 42 °C, the membrane was washed twice as follows: (a) 5 min at room temperature with 2× SSC, 0.5% SDS; (b) 30 min at 65 °C with 2× SSC, 0.5% SDS, and finally; (c) 30 min at room temperature with 0.1× SSC. An optional stringent washing with 0.3× SSC could be performed at 65 °C, which however, reduces the hybridization signals. The membrane was briefly blotted between paper towels and while still damp, wrapped in Saran and exposed to Kodak XAR-5 films at −70 °C in presence of an intensifying screen. The developed film was scanned with a Joyce-Loebl MK IIIc microdensitometer and peak areas were measured with a Numonics Corporation Electronic Graphics Calculator.

Erasing of the probe

The ^{32}P-labeled DNA probe was stripped from the membrane as described (10, 18), with the following minor modifications. After each round of prehybridization-hybridization and autoradiography, the damp membrane was washed with constant agitation with a solution of 75% deionized formamide in 0.1× SSC for 2 h at 75 °C, rinsed twice in 0.1× SSC to remove excess formamide, once in distilled water and then air dried at room temperature. The membrane could be reused immediately or stored under vacuum.

Preparation of the ^{32}P-labeled DNA probes

Full-length plasmids were labeled by nick translation (20). The reaction was stopped by adding 200 μl of a solution containing 0.5 M sodium chloride, 10 mM EDTA and 0.5% SDS (18) and the labeled DNA was isolated by chromatography through a Sephadex G-100 (Pharmacia) column.

The following plasmids were used in this study: pMmH4, mouse histone H4 gene (22); pmycEcl (a subclone of a human c-myc gene established by P. Martin and D. Stehelin; O. Brison *et al.*, unpublished); pMrSalB, the ETS 5′-end of mouse 45S pre-rRNA (11); pX-R1, human heat shock cognate hsc 70 gene (M.-E. Mirault and B. Dworniczak, unpublished); pDHFR11, mouse dihydrofolate reductase gene (23); pDmA, Drosophila actin gene (8) and BLUR8, human alu-DNA (6). Polyoma A2 DNA was prepared as described (12).

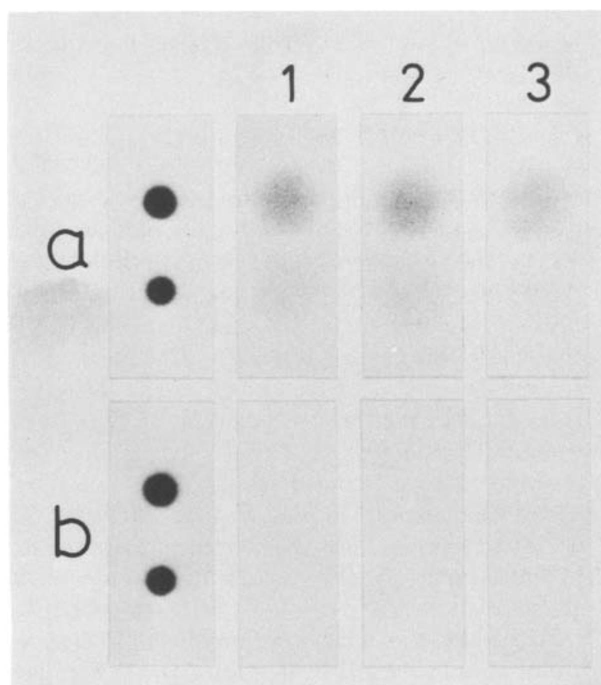


Fig. 1. Demonstration of the retention of RNA on NC and GS membranes. One μg of [^{32}P] RNA (10000 cpm) in 15 μl was denatured with 16.5% formaldehyde at 65 °C for 15 min and diluted with an equal volume of 20× SSC. Thirthy μl aliquots containing 1μg or 0.5 μg RNA were applied to NC or GS membranes cushioned on three layers of filter papers. The membranes as well as the filter papers were dried and exposed to Kodak XAR-5 films for 24 h at −70 °C with an intensifying screen. (a) nitrocellulose; (b) gene screen membranes, respectively; 1 to 3 refer to the three successive filter papers on which the membranes were layed on during the dot-blotting.

Results and discussion

Retention of RNA to the nylon membranes

Preliminary experiments were performed to compare the retention of RNA to nitrocellulose (NC) and nylon Gene Screen (GS) membranes. Aliquots of denatured ^{32}P-labeled RNA were spotted on either NC or GS using a manifold apparatus. It was noted that when applied under even slight vacuum, variable amounts of radioactivity were recovered into the lower tank of the apparatus, substantiating that rapid flow through the membrane is inadequate for quantitative analysis. However, when the [^{32}P]RNA was drawn through the membrane by absorption alone, NC retained 60–70% of the ^{32}P-label, while the remainder migrated through three or more layers of filter papers (Fig. 1a). In contrast, GS membranes retained 95% of the ^{32}P-label, determined by autoradiography (Fig. 1b) and liquid scintillation counting.

Conditions of RNA hybridization

When GS membranes were processed for hybridization according to the procedure developed for NC by Thomas (26), an autoradiographic image referred to as 'black-blot' was obtained. Hybridization signals were scarcely detected since an intensive background was observed. The same high background was observed whether the RNA was transferred by the capillary blot (24) or by the electrophoretic transfer procedures. Since nylon-based membranes have a considerably higher binding capacity for anionic molecules than NC (9), GS nonspecifically adsorbs the labeled probes used for hybridization, thereby producing strong backgrounds.

To increase the intensity of the signals and to decrease the nonspecific background, the five following steps are critical during prehybridization of GS: (1) the use of a concentrated blocking solution (10× Denhardt's solution; ref. 7), and of ionic detergents; (2) prehybridization for at least 16 h; (3) filtration through a 0.45 μm HA-Millipore of the prehybridization solution and; (4) the volume of the prehybridization solution which should be at least 0.13 ml per cm^2 of membrane. Finally, because of the high viscosity of the solution due to the presence of dextran sulfate, constant rocking at low frequency (1–2 cycles per min) is necessary to permit a regular and constant flow over the membrane[1].

Sequential hybridization of RNA on nylon membrane

Using the conditions described above a series of sequential hybridizations with several probes were performed on the same GS to determine the sizes of different RNAs (Fig. 2). Since the same membrane was used throughout these hybridizations, differences in electrophoresis and electrophoretic transfer conditions were eliminated. Successive hybridizations to the same nylon membrane resulted in a gradual loss of RNA from the matrix. Therefore, experiments were planned so that hybridizations to abundant transcripts followed those to rare ones (10).

As representative of these analyses, hybridization data with DHFR mRNA is presented here. A major species of 1.6 kb, and minor ones of approximately 4.0, 1.2, and 1.0 kb were detected. The sensitivity obtained with as little as 5 μg of total cytoplasmic RNA was comparable to that achieved on DBM paper with poly(A)$^+$ mRNAs selected from methotrexate-resistant cells that overproduce DHFR mRNAs (23).

UV crosslinking of RNA to nylon membrane

Five different methods of RNA fixation to nylon membranes were compared by evaluating the intensity of the hybridization signals. Five aliquots, each containing 1 μg of denatured total RNA, were electrophoresed in parallel in formaldehyde-agarose gel, and the RNA electrophoretically transferred to a GS membrane. Five individual strips containing one electrophoretic track each were cut out from the membrane and the RNA fixed onto it according to one of the following protocols: (1) exposure to an infra-red, Infraphil 150W-2K lamp (Philips) at a distance of 40 cm for 10 min; (2) baking at 65 °C

[1] A prehybridization solution containing 1 M sodium chloride, 50 mM Tris-HCl pH 7.5, 1% SDS, 10% dextran sulfate, 50% formamide and 0.5% dry nonfatty milk (Johnson *et al.*, Gene Anal. Techn. 1: 3–8, 1984) was effective in blocking the GS membrane. However, a background was observed after prolonged autoradiography of the membranes. For this reason the dry milk was not used for the detection of rare transcripts.

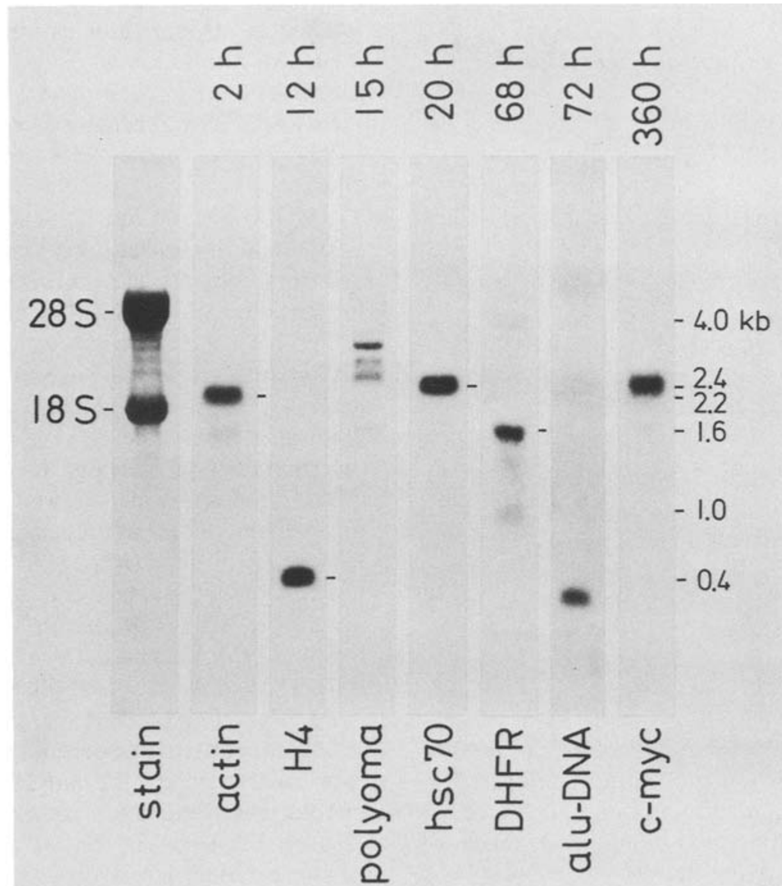


Fig. 2. Sequential hybridizations with different probes to the same GS membrane. Five μg of total cytoplasmic RNA were electrophoresed, electrotransferred and hybridized as described in the text. After each round of hybridization and autoradiography, the signals were erased and the same membrane hybridized with the next probe. The probes used in each hybridization assay to detect homologous RNAs are indicated below, and exposure time of the corresponding autoradiograph above each lane.

for 2 h; (3) baking at 80 °C for 2 h without vacuum (18); (4) baking at 80 °C for 2 h under vacuum, and finally, (5) exposure to UV irradiation for 2 min (5). The five strips were processed in the same bag for hybridization with a nick translated plasmid containing a mouse histone H4 DNA insert. The results obtained after autoradiography (Fig. 3) showed that hybridization signals were detected on all five GS membranes, although the intensity of the hybridization signals were not the same.

Taking the results from treatment 4 (baking at 80 °C under vacuum, Fig. 3d) as a standard value, the ratios of signals quantitated by liquid scintillation counting of the excised membrane areas and by densitometric scanning of the films were: 0.4 (Fig. 3a), 0.5 (Fig. 3b), 0.9 (Fig. 3c) and 10.0 (Fig. 3e), as determined in 4 independent experiments. Exposure of the membrane to UV irradiation for more than 2 min, resulted in decrease hybridization signals[2].

The results suggest tht the increased intensity of the radioactive signals obtained after short UV irradiation may be the consequence of a more stable fixation of RNA to the membrane, a situation simi-

[2] We have observed that optimal hybridization signals were obtained when the membrane was UV irradiated while still damp. Damp membranes are uniformally matt without any glossy area and remain flat. UV irradiation of dried membranes, which were curled, resulted in hybridization signals comparable to those obtained after fixing the RNA by backing at 80 °C.

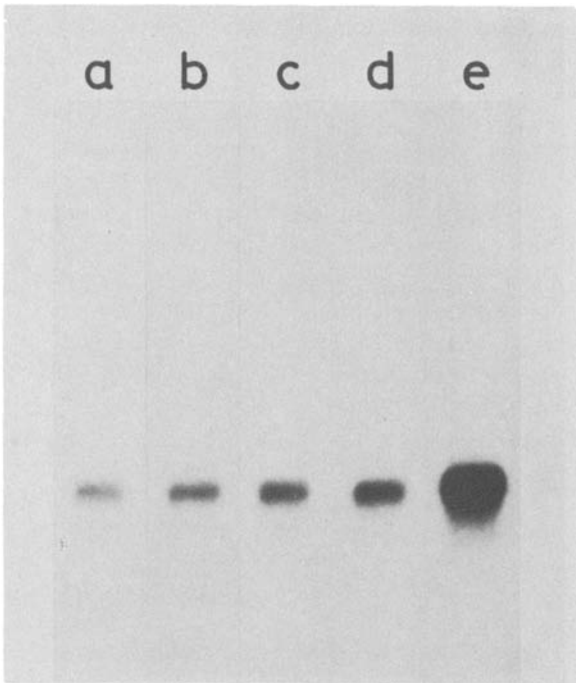


Fig. 3. Levels of hybridization signals depend on fixation of RNA to GS membrane. Five aliquots each containing one μg of total RNA were electrophoresed in parallel in a formaldehyde-agarose gel and RNA electrotransferred to GS membrane. Each individual strip was treated as follows: exposure to an Infra-red lamp (lane a), baking at 65 °C (lane b), baking at 80 °C without vacuum (lane c), baking at 80 °C under vacuum (lane d), and exposure to UV irradiation (lane e). For details see Results. The five strips were processed in the same bag for hybridization with a nick translated pMmH4 plasmid containing a mouse histone H4 DNA insert. Autoradiography at −70 °C was for 48 h.

lar to that described for DNA crosslinked to the same type of nylon membrane (5); alternatively, UV irradiation might either stabilize secondary structures or, induce conformational changes of the RNAs which would increase hybridization affinity to complementary DNA sequences.

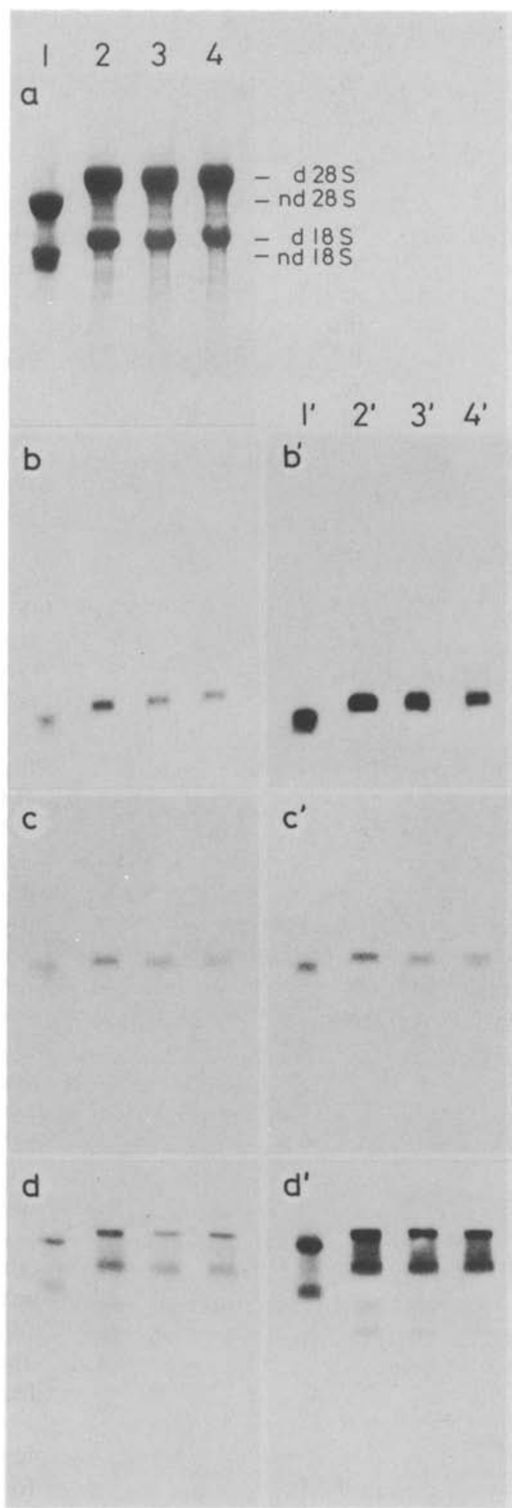
Retention and hybridization of denatured and non-denatured RNA on nylon membranes after UV irradiation

For efficient transfer from agarose gels to NC membranes RNA has to be denatured (26). We compared the efficiency of the electrotransfer, the binding of RNA to nylon membranes and the intensity of the hybridization signals of denatured and non-denatured RNA. Aliquots of 5 μg total RNA were denatured by heating at 65 °C for 5 min in one of the following solutions: (a) 1× MOPS buffer, 10% formaldehyde, (b) 1× MOPS, 8% formaldehyde, 25% formamide or, (c) 1× MOPS, 6% formaldehyde, 50% formamide; (d) as non-denatured controls 5 μg RNA were suspended in 1× MOPS without heating. Duplicates of the RNA samples were electrophoresed in a formaldehyde-agarose gel and electrotransferred to a GS membrane. One half of the GS membrane was baked at 80 °C under vacuum (series a–d), while the other half was UV irradiated for 2 min (series b′–d′). As determined by staining with methylene blue, the amounts of 28S and 18S rRNA present on the GS membranes were independent of the pretreatment of the RNA and the mode of fixation (Fig. 4a). In accordance with earlier reports (14, 25) non-denatured 28S and 18S rRNA exhibited a higher mobility (Fig. 4a, lane 1). However, only small differences were observed between the mobility of the rRNAs denatured with formaldehyde either in the absence (Fig. 4a, lane 2) or the presence of 25% (lane 3) or 50% (lane 4) formamide.

Subsequently, the membranes were sequentially hybridized with ^{32}P-labeled DNA probes for H4 mRNA (Fig. 4b and b′), actin mRNA (Fig. 4c and c′) and 45S pre-rRNA (Fig. 4d and d′). Again a decrease in mobility was observed in formaldehyde-denatured RNA preparations which was slightly more pronounced if denaturation has taken place in the presence of 25% or 50% formamide. As determined in repeated experiments, the radioactive signals were 4 times higher with RNA preparations that had been heat-denatured with formaldehyde alone (Fig. 4, lanes 2, 2′ and 4, 4′).

To compare hybridization signals of RNA fixed by baking or UV-irradiation, the GS membrane halves were hybridized in the same bag. The results showed that fixation of RNA by UV-irradiation increased the radioactive signals for H4 mRNA, actin mRNA and 45S pre-rRNA by a factor of 10, 3 and 5. These results were highly reproducible and indicate that structure and/or base competition of the RNAs, rather than their molecular weights, affect their crosslinking by UV.

Our observations also indicate that complete heat-denaturation of the RNA in presence of formaldehyde and formamide, although prerequisite for the determination of molecular weights (14, 25),



is not required for stable binding of RNA to GS membranes either after baking or UV-irradiation. In all subsequent experiments we therefore used the following standard procedure: RNA samples were denatured at 65 °C for 5 min in the presence of 6% formaldehyde in 1× MOPS buffer pH 7.0; the samples were then electrophorezed in formaldehyde-agarose gel, transferred to GS membrane and the RNA fixed by UV-irradiation for 2 min.

The use of GS membranes for combined analysis of synthesis and steady state levels of RNAs

Primary mouse kidney cell cultures were pulse-labeled with [5-^{3}H]-uridine for 1 h, and total RNA was extracted with phenol. Aliquots containing 5 μg RNA were heat-denatured under the standard conditions, subjected to electrophoresis in formaldehyde-agarose gel, electrotransferred to a GS membrane and the RNA fixed by UV-irradiation. Staining with methylene blue revealed a faint band corresponding to 45S pre-rRNA and major bands corresponding to 28S and 18S rRNA (Fig. 5a). Fluorography of the membrane, revealed as expected most of the radioactivity between the 45S pre-rRNA and the 32S intermediate (Fig. 5b). After washing the GS membrane with ethanol to remove the PPO, it was hybridized with the DNA probe containing the 5'-terminus of mouse 45S pre-rRNA which is rapidly removed during pre-rRNA maturation (19). Autoradiography for 5 min only (Fig. 5c) revealed 2 major radioactive bands corresponding to 45S pre-rRNA and 32S intermediate. If exposure

Fig. 4. Sequential hybridizations of different probes to size-fractionated RNA transferred to GS membrane and fixed after baking at 80 °C in vacuum (left panels, a, b, c and d) or after UV irradiation (right panels, b', c' and d'). In the same experiment, four different conditions of RNA preparation were also tested. Aliquots of 5 μg of total RNA were heated at 65 °C in presence of: 1× MOPS buffer, 6% formaldehyde, 50% formamide (tracks 4 and 4'), 1× MOPS, 8% formaldehyde, 25% formamide (tracks 3 and 3'), 1× MOPS, 10% formaldehyde (tracks 2 and 2'). As control, RNA in 1× MOPS was not heat denatured (tracks 1 and 1'). Methylene blue stained membrane (a). Autoradiography after sequential hybridizations with the following nick translated plasmids: pMm H4 (histone H4 DNA, panels b and b', exposure 12 h), pDmA (actin DNA, panels c and c', exposure 4 h), pMrSalB (ETS 5'-end of mouse 45S pre-rRNA, panels d and d', exposure 30 min).

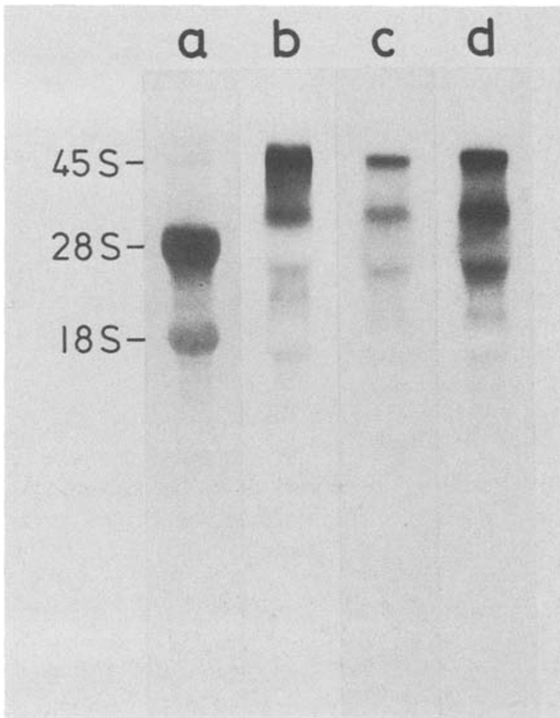


Fig. 5. Analysis of newly synthesized RNA followed by Northern analysis. Five μg of [^{3}H]-labeled total RNA were size-fractionated on formaldehyde-agarose gel, electrotransferred to GS membrane and UV irradiated. After staining with methylene blue (lane a), the same membrane was processed for fluorography (exposure for 15 days to a XAR-5 film, lane b), followed by hybridization with nick translated pMrSalB (mouse 45S pre-rRNA) plasmid. The membrane was exposed at room temperature to XAR-5 films with an intensifying screen for 5 min (lane c), and for 2 h (lane d).

was extended to 2 h (Fig. 5d), additional radioactive bands were observed in the size range extending between 26S to 10S; these bands possibly correspond to cleavage products of 45S pre-RNA maintaining their 5′-terminus (3). No hybridization signals were observed with the 28S and 18S rRNA. This method is thus potentially useful to study simultaneous synthesis and processing of cellular RNA species.

Conclusion

The results reported here are an improved version of the Northern blot analysis. The radioactive signals of mRNAs and ribosomal precursors RNAs are 10 to 40 times increased and the non-specific background decreased. The method allows to measure steady state levels of minor mRNA species in cytoplasmic or total RNA without the necessity to isolate poly(A)$^+$mRNAs. Since the amounts of RNA present on the GS membranes used for hybridization can be measured by staining with methylene blue, the methods allowed us to perform precise time course studies on the expression of a variety of viral and cellular genes. In addition the method reported here is useful to determine on a single membrane apparent rate of synthesis and steady state levels of different RNA species.

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