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Method for detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethyl-paper and hybridization with DNA probes

(single-stranded nucleic acids/methyl mercuric hydroxide-agarose gels/*Drosophila melanogaster* RNA/hybrid plasmids)

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ABSTRACT **We describe a technique for transferring electrophoretically separated bands of RNA from an agarose gel to paper strips. The RNA is coupled covalently to diazobenzyloxymethyl groups on the paper. After transfer and appropriate treatment of the paper to destroy remaining diazo groups, specific RNA bands can be detected by hybridization with ^{32}P-labeled DNA probes followed by autoradiography. This procedure allows detection of specific RNA bands with high sensitivity and low background.**

The technique of Southern (1) enables one to transfer to nitrocellulose strips electrophoretically separated DNA species or fragments of DNA generated by cleavage with restriction endonucleases. Specific bands within an array can be detected subsequently by autoradiography, after hybridization with specific radioactive RNA or DNA (2). This procedure has become routine, for example, in the mapping of specific sequences in viral genomes and DNA segments that have been cloned in hybrid plasmid or viral vectors. For many experiments it would be useful to reverse the technique, i.e., to transfer electrophoretically separated RNA species to a solid support and detect specific species by hybridization with radioactive complementary DNA or RNA. However, RNA does not in general bind to nitrocellulose. Noyes and Stark (3) coupled single-stranded DNA or RNA covalently to finely divided cellulose that had been derivatized with diazobenzyloxymethyl groups and used this as an insoluble support for hybridization. We have prepared diazobenzyloxymethyl (DBM)-paper and developed procedures by which single-stranded nucleic acid can be transferred from a gel and coupled covalently to the paper. Specific sequences are detected with high sensitivity and low background by hybridization to specific nucleic acid probes labeled with ^{32}P.

MATERIALS AND METHODS

Synthesis of 1-[(*m*-Nitrobenzyloxy)methyl] Pyridinium Chloride. 1-[(*m*-Nitrobenzyloxy)methyl] pyridinium chloride (NBPC) was synthesized according to Kursanov and Solodkov (4). Dry HCl (454 g) was bubbled into a solution containing 158 g of paraformaldehyde and 200 g of *m*-nitrobenzyl alcohol (Aldrich Chemical Co.) in 1 liter of benzene for 2 hr at room temperature with stirring, and stirring was continued overnight. After settling, the upper organic phase was removed and dried with 150 g of anhydrous Na_2SO_4 and the benzene was removed under reduced pressure. The remaining yellow liquid was distilled under reduced pressure and the fraction boiling between 150 and 154° at 1.5 mm of Hg was collected, yielding 216 g of yellow liquid. This material was added slowly to 750 ml of ice-cold pyridine with stirring, and the pyridinium salt was allowed to crystallize. It was collected on a sintered glass filter, washed with pyridine, then washed thoroughly with petroleum ether and dried under reduced pressure. The NBPC (267 g) was stored at −20° in a desiccator.

Preparation of Aminobenzyloxymethyl-Paper. The method for making aminobenzyloxymethyl (ABM)-paper and its subsequent conversion to the diazobenzyloxymethyl form (DBM-paper) by diazotization is outlined in Fig. 1. The ABM-paper was prepared by a modification of methods described previously (5–7) for preparing aminobenzyloxymethyl-cellulose powder. A sheet of Whatman 540 paper (14 × 25 cm) in a flat enameled pan was soaked with 10 ml of an aqueous solution of 0.8 g of NBPC and 0.25 g of sodium acetate. Air bubbles under the paper were squeezed out and the paper was dried at 60° and then heated to 130–135° for 35 min. The paper was washed twice for 20 min with water, dried at 60°, washed twice for 20 min with benzene, and dried in the air. The nitrobenzyloxymethyl paper was reduced to ABM-paper by treating it with 150 ml of 20% sodium dithionite (wt/vol) for 30 min at 60° with shaking. The ABM-paper was washed for 20 min with water, 20 min with 30% acetic acid, and then with water until there was no further odor of H_2S. The ABM-paper was dried in the air and stored at 4° in a desiccator. It is stable for several weeks under these conditions.

Diazotization of ABM-Paper. Just before reaction with single-stranded nucleic acids, ABM-paper was converted to the diazobenzyloxymethyl (DBM) form by treatment with a solution containing 40 ml of water, 80 ml of 1.8 M HCl, and 3.2 ml of a freshly prepared solution of $NaNO_2$ (10 mg/ml) for 30 min at 4°. The solution was checked for free HNO_2 with starch-iodide paper, which turns black. After 30 min, the DBM-paper was washed five times for 5 min each with 100 ml of cold water and then twice for 10 min with ice-cold sodium borate buffer, 50 mM, pH 8. Upon washing, the paper turns bright yellow. It should be kept cold until transfers begin, no more than 15 min later. DBM-paper had the capacity to couple 16–24 μg of single-stranded nucleic acid per cm^2 of surface area.

Gel Electrophoresis in the Presence of Methyl Mercuric Hydroxide. Nucleic acid samples were separated by electrophoresis on horizontal slab gels (23 × 14 × 0.4 cm) containing 1.5% agarose and 4 mM methyl mercuric hydroxide (Alfa) according to Bailey and Davidson (8). Samples in water were mixed with an equal volume of starting buffer [1× E buffer (8)/10% glycerol/bromophenol blue] and made 10 mM in methyl mercuric hydroxide. Samples were run at 15–25 V (measured across the gel) for 17–20 hr. The gels were prepared for transfer as described in *Results and Discussion*.

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Abbreviations: NBPC, 1-[(*m*-nitrobenzyloxy)methyl] pyridinium chloride; ABM, aminobenzyloxymethyl; DBM, diazobenzyloxymethyl.

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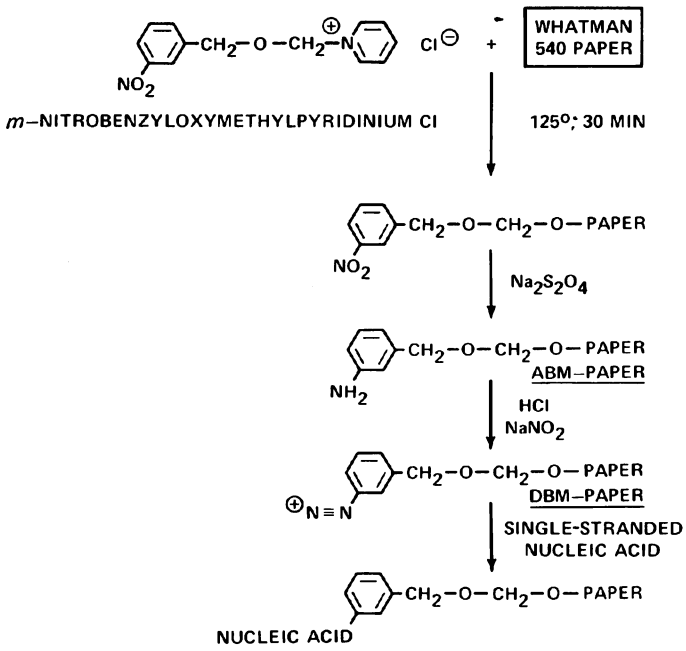

FIG. 1. The sequence of reactions involved in making DBM-paper and in coupling single-stranded nucleic acids to it.

Preparation of RNA from *Drosophila melanogaster* Cells and Tissues for Gel Electrophoresis. All operations were at 0–4°, with siliconized glassware. The method described was scaled up as necessary. Up to 0.1 ml of packed cells were suspended in 0.5 ml of Robb's saline (9). The suspension was then pipetted into the center of a mixture being stirred on a Vortex mixer containing 2.0 ml of extraction buffer (0.1 M NaCl/0.1 M Tris·HCl/30 mM Na_2EDTA/1% wt/vol Sarkosyl at pH 8.9) and 1.0 ml of phenol that had been equilibrated against 10 mM Tris·HCl, pH 8.1. After the mixture was on a Vortex mixer for 1 min, 1.0 ml of $CHCl_3$ containing 4% vol/vol isoamyl alcohol was added and the mixture was again mixed for 1 min. After centrifugation of the mixture at 10,000 rpm for 1 min, the upper aqueous phase was withdrawn and mixed with 1 volume of phenol. The organic phase was re-extracted in the same manner with extraction buffer (1.0 ml). The resulting aqueous phase was pooled with the first aqueous phase and re-extracted first with phenol/$CHCl_3$/isoamyl alcohol as described above and then with $CHCl_3$/isoamyl alcohol. RNA was precipitated at −20° for 16–24 hr after addition of 0.1 volume of 1.0 M sodium acetate (pH 5.0) and 2.5 volumes of ethanol, collected by centrifugation, dried under reduced pressure at room temperature, dissolved in a solution containing 10 mM Tris·HCl/1 mM EDTA at pH 7.4, and disaggregated by heating at 80° for 1 min.

Such RNA preparations also contain DNA, but this accounts for only a minor fraction of the total nucleic acid in *D. melanogaster* cells.

***D. melanogaster* Cells and Tissues Used for RNA Preparation.** Salivary glands were isolated from late third instar larvae of *D. melanogaster* by a modification (D. J. Kemp and D. S. Hogness, unpublished) of the procedure of Zweidler and Cohen (10). *D. melanogaster* embryos (0–17 hr) were dechorionated and then homogenized in a glass–Teflon homogenizer at 0° in 185 mM sodium chloride. The homogenate was then used immediately for the preparation of RNA as described above. The RNA from Eschalier's Kc_0 line of cultured cells was labeled with [^{32}P]orthophosphate for 13 hr as described by Rubin and Hogness (11).

Hybrid Plasmid DNAs. Plasmid DNAs, isolated as described by Wensink *et al.* (12) with minor modifications, were labeled with ^{32}P by nick translation with DNA polymerase I as described by Rigby *et al.* (13). The hybrid plasmid cDm 103 consists of a 17,000-base-pair repeat unit of *D. melanogaster* rDNA inserted into the ColE1 plasmid (14); it contains a complete complement of sequences homologous to those in the 18 and 28S rRNAs (15). The hybrid plasmid pkdm 34-H-9 contains sequences homologous to *D. melanogaster* 14S mitochondrial RNA. It was constructed by inserting into the *Bam* HI site of the pSC105 plasmid (16, 17) a duplex cDNA segment obtained by reverse transcription of the 14S RNA, using a modification of the procedure of Maniatis *et al.* (18) developed for the rapid cloning of cDNA segments (D. J. Kemp and D. S. Hogness, unpublished).

Containment Conditions. Plasmids containing *D. melanogaster* segments were propagated by EK1 host–vector systems under P2 physical containment, as defined and recommended for this class of experiments by "National Institutes of Health Recombinant DNA Research Guidelines" (*Federal Register*, July 7, 1976).

RESULTS AND DISCUSSION

Preparation of Gels for Transfer. In order to transfer single-stranded nucleic acids from a methyl mercuric hydroxide-agarose gel to DBM-paper, several problems must be overcome. Since methyl mercuric hydroxide, a powerful denaturing agent, reacts with nitrogen atoms in the bases of nucleic acids (8), it must be removed before transfer to allow the bases to react with the diazotized paper. Some reagents used to remove bound methyl mercuric hydroxide, e.g., ammonium ions (8), would also react with diazonium groups. We therefore used a low concentration of 2-mercaptoethanol to remove the methyl mercuric hydroxide from the gel and the nucleic acid. The gel was then washed with excess iodoacetate to remove the 2-mercaptoethanol, and then with dilute borate buffer to remove the iodoacetate and to provide an ionic environment appropriate for the transfer. We found that RNA species ≥18 S will not transfer efficiently under these conditions. To overcome this problem, we partially degraded the RNA in the gel by limited alkaline hydrolysis under conditions that reduced the size of 18 and 28S rRNAs enough to allow them to transfer from the gel but did not appreciably lower the retention of tRNA by DBM-paper.

The following protocol was developed to satisfy the above considerations. After electrophoresis (see *Materials and Methods*), the gel was placed in 200 ml of 50 mM NaOH containing 5 mM 2-mercaptoethanol and rocked gently for 40 min at room temperature (22–25°). The gel was then treated twice with 200 ml of 200 mM sodium borate (pH 8.0) containing 7 mM iodoacetic acid for 10 min at room temperature and then twice with 200 ml of 50 mM sodium borate (pH 8.0) for 5 min at room temperature. If the gel did not contain methyl mercuric hydroxide, the 2-mercaptoethanol and iodoacetate were omitted from the sodium hydroxide and the borate, respectively. If alkaline hydrolysis was not desired, for example, in the study of small RNA species such as tRNA where hydrolysis does not improve transfer, then the NaOH was replaced by electrophoresis buffer containing mercaptoethanol. If transfer from a gel that does not contain methyl mercuric hydroxide was desired without the alkaline hydrolysis, the gel was simply washed in 50 mM borate (pH 8.0) before transfer.

We have not investigated conditions for staining the RNA bands before transfer. If staining of markers is required, then these are run in separate tracks, which are excised from the gel

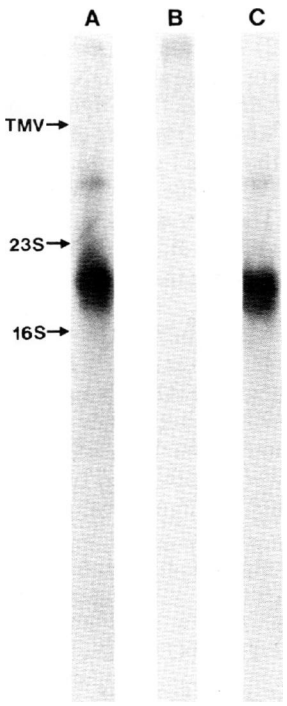

FIG. 2. Transfer of ^{32}P-labeled RNA)specific activity 9.7×10^4 cpm/μg) of *D. melanogaster* Kc cells to DBM-paper. (*A*) The pattern of bands in the gel; (*B*) the pattern of bands remaining in the gel after transfer; (*C*) the pattern of bands transferred from the gel and covalently bound to DBM-paper. The patterns were visualized by autoradiography for 20 hr. The positions of *Escherichia coli* 23S and 16S ribosomal RNAs and tobacco mosaic virus (TMV) RNA, run on the same gel and detected by staining with ethidium bromide as described (8), are shown by arrows.

and stained as described (8). We found that RNA bands close to the cut edge of the gel can develop severe distortions during transfer unless at least 0.5 cm is left between the cut edge and the nearest RNA track.

Transfer of RNA from the Gel to DBM-Paper. After treatment as above, the procedure for transfer is like that described by Southern (1). The gel was placed on two sheets of Whatman 3MM paper saturated with 50 mM sodium borate buffer (pH 8.0) and a strip of DBM-paper saturated in this buffer was placed directly on the gel, using Plexiglas strips to prevent the DBM-paper from contacting the underlying 3MM paper at the sides of the gel. The DBM-paper was then covered with two layers of dry 3MM paper, several layers of paper towels, and finally a Plexiglas weight. The borate buffer was allowed to blot through until the towels were soaked. After four changes of towels the assembly was left overnight. It is essential to time the conversion of ABM-paper to DBM-paper so that the latter can be placed on the gel within a few minutes after the final gel washes are complete.

The transfer of ^{32}P-labeled *D. melanogaster* RNA from a methyl mercuric hydroxide gel to DBM-paper is shown in Fig. 2. In Fig. 2*A*, which shows the autoradiograph of a gel track containing an electrophoretically fractionated sample of [^{32}P]RNA, a broad major 18S band migrating between the 16 and 23S markers and a minor 28S band migrating between the 23S and tobacco mosaic virus markers are apparent. This pattern results because *D. melanogaster* 28S rRNA is rapidly cleaved *in vivo* at a specific site near its center (19) and the two resulting fragments, which are dissociated under the electrophoretic conditions used here, migrate just in front of and behind the 18S rRNA, respectively (20). The autoradiograph has been overexposed in order to detect the small amount of intact 28S [^{32}P]rRNA; consequently, the three species migrating around 18 S, which are easily resolved at lower exposures, are not detected here.

Fig. 2*B* shows a gel track identical to that in Fig. 2*A*, except that the RNA was transferred from it as described above. Most of the RNA has been removed from the gel during transfer. The faint smear at the top of the gel is primarily DNA, as shown by its susceptibility to DNase, and presumably is too large to transfer. Fig. 2*C* shows an autoradiograph of the DBM-paper strip to which the RNA removed from this gel was transferred. By comparison with Fig. 2*A* it is clear that a major proportion of both the 18 and 28S rRNA removed from the gel has bound to the paper. The proportion bound, determined by scintillation counting of the excised bands, varied somewhat in different experiments but was usually about 30–60% of the total. Unmodified paper or ABM-paper did not bind significant amounts of RNA.

We think that reaction of single-stranded nucleic acids with the paper proceeds first through a noncovalent interaction with the positive charges on the diazonium groups, similar to the interaction of nucleic acid with DEAE-cellulose, followed by the covalent coupling. The postive charges are eventually lost by hydrolysis or replaced by negative charges of the carrier DNA and glycine used in the prehybridization treatment of the paper (see below). While we have not shown directly that the coupling is covalent, the RNA is not removed by washing with 99% formamide, H_2O, or the hybridization buffer.

We have not investigated the conditions required for effective transfer from gels whose thickness or concentration of agarose differs from those described in *Materials and Methods*. The time required for alkaline hydrolysis and removal of reagents may vary considerably, depending on rates of diffusion in different gels.

Hybridization with Specific Radioactive Probes. ^{32}P-Labeled plasmid DNA probes were hybridized to RNA on the paper by a procedure similar to that used by Denhardt (2) for hybridizing DNA immobilized on nitrocellulose filters with a radioactive DNA probe. The paper strips containing transferred RNA were treated for 4–24 hr at 42° with hybridization buffer (50% formamide/0.75 M sodium chloride/75 mM sodium citrate containing 0.02% wt/vol each of bovine serum albumin, ficoll, and polyvinylpyrrolidone, 1.0–2.5 mg of sonicated denatured calf thymus or salmon sperm DNA per ml, and 1% wt/vol glycine) in order to hydrolyze any remaining diazo groups and block any other nonspecific sites on the paper that might bind the ^{32}P-labeled DNA probe. Hybridizations were performed by placing the paper strips, hybridization buffer minus glycine (50–100 μl/cm^2 of paper surface area), and the single-stranded ^{32}P-labeled probe (2×10^4–2×10^5 cpm/cm^2 of paper surface area; specific activity 2 to 6×10^7 cpm/μg) into plastic boiling bags (Seal-N-Save, Sears) from which the excess air was removed prior to sealing by heat. The bags were laid horizontally and rocked gently for 36 hr at 42°. The paper was then washed at 42° for at least 4 hr with at least six changes of a solution containing 50% formamide/0.75 M sodium chloride/75 mM sodium citrate. The paper strips were blotted to remove excess solution, covered with Saran wrap, and visualized by autoradiography with Kodak XR-5 x-ray film.

If the plasmid DNA probe contained a dA·dT connector (12) (or if some other DNA probe were to contain other dA·dT-rich regions) the pretreatment and hybridization mixtures contained, in addition, 0.5 mg of poly(U) per ml, to compete with the potential hybridization of poly(dT) in the probe to the poly(A) segments in the mRNA attached to the paper.

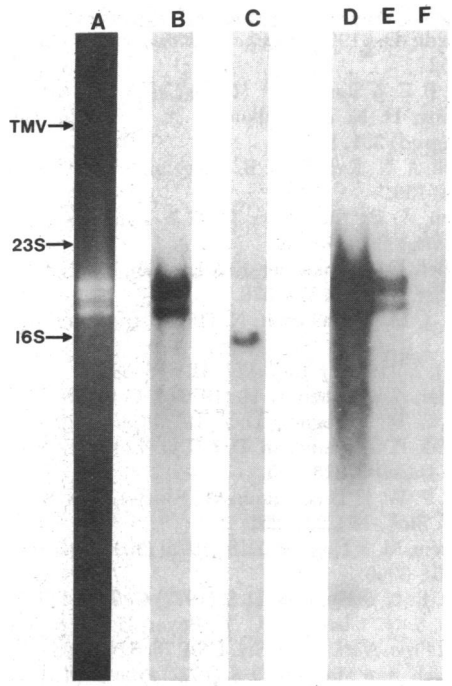

FIG. 3. Transfer of *D. melanogaster* RNA to DBM-paper and visualization of specific bands by hybridization to specific ^{32}P-labeled DNA probes. (*A*) Pattern of 1 μg of salivary gland RNA after staining with ethidium bromide. (*B*) Autoradiographic pattern of bands visualized after hybridization with cDm 103 [^{32}P]DNA (2×10^4 cpm/cm^2 of paper surface area; specific activity, 2×10^7 cpm/μg); 100 ng of salivary gland RNA was run on the gel. (*C*) Transfer of *D. melanogaster* embryonic RNA and autoradiographic visualization after hybridization with a fragment of pkdm 34-H-9 [^{32}P]DNA (2×10^4 cpm/cm^2 of paper surface area; specific activity 6×10^7 cpm/μg). The fragment was generated from pkdm 34-H-9 DNA by gel electrophoresis after digestion with the restriction endonuclease *Sal* I and contained the *D. melanogaster* DNA segment; 10 μg of *D. melanogaster* embryonic RNA was run on the gel. (*D*) *D. melanogaster* salivary gland RNA (100 ng) was run on the gel, transferred, and hybridized with cDm 103 [^{32}P]DNA as in *B*. (*E* and *F*) Identical to *D* except that 10 ng and 1 ng, respectively, of *D. melanogaster* salivary gland RNA were run on the gels. The exposure times for the autoradiographs were: *B*, 5 hr; *C–F*, 16 hr. The markers are the same as those indicated in Fig. 2.

Fig. 3*A* shows a gel track of *D. melanogaster* salivary gland RNA that has been stained with ethidium bromide. The three rRNA bands described above are clearly visible. When this RNA was transferred to a DBM-paper strip and hybridized with cDm 103 [^{32}P]DNA, the autoradiograph shown in Fig. 3*B* was obtained. Since the plasmid cDm 103 contains a complete complement of sequences homologous to *D. melanogaster* 18 and 28S rRNAs (see *Materials and Methods*), it was predicted that the cDm 103 [^{32}P]DNA should hybridize only to these rRNAs, and that is clearly the result observed in this autoradiograph.

The three radioactive bands shown in Fig. 3*B* correspond to the most abundant species present in the RNA applied to the gel, and one might imagine that they result from nonspecific binding of the probe. To discount this possibility we hybridized a similar DBM-paper strip containing embryonic RNA with pkdm 34-H-9 [^{32}P]DNA. This plasmid contains sequences homologous to *D. melanogaster* 14S mitochondrial RNA, which is 1500 bases in length and constitutes between 0.25 and 0.5% of the mass of embryonic RNA (D. J. Kemp and W. J. Peacock, unpublished). The stained gel pattern for embryonic RNA (data not shown) was essentially the same as that for salivary gland RNA shown in Fig. 3*A*. Fig. 3*C* shows that pkdm 34-H-9 [^{32}P]DNA labeled a single band with mobility close to that of the 16S *E. coli* rRNA marker, as predicted. By contrast, the three rRNA bands, which together constitute >90% of the mass of the RNA, were not detectably labeled. We have also obtained the predicted pattern of labeled bands when a plasmid containing the *D. melanogaster* histone genes was used as a probe (data not shown). We conclude that the labeled bands represent sequence-specific hybridization.

The sensitivity of the procedure was investigated in the experiments shown in Fig. 3*D–F*. These show autoradiographs of tracks similar to that in Fig. 3*B* after hybridization with the same cDm 103 [^{32}P]DNA probe except that the amounts of salivary gland RNA used was progressively decreased and the exposure time increased. In Fig. 3*D* the major rRNA bands are now overexposed and a smear of smaller material, presumably degraded rRNA, is obvious. A small discrete band near the bottom of the gel was reproducibly labeled and may represent 5.8S RNA, although we could not accurately determine its size with the markers we used. Fig. 3*F* shows that the three rRNA bands can still be detected even when each derives from only 300 pg of RNA. We note that this track contained $1/10^4$ the rRNA of that present in the track shown in Fig. 3*C*, where none of the rRNA was labeled by the specific mitochondrial RNA probe.

Concluding Remarks. We have developed a rapid and sensitive gel-transfer hybridization technique for detecting and determining the size of specific RNA molecules. DBM-paper strips are easy to handle and can be reused with another probe after they are washed in 99% formamide at 65° to remove the first probe. If the alkaline hydrolysis gel treatment described is used, the size of the RNA molecule makes little difference to the efficiency of the transfer. We have shown that RNA molecules varying in size from tRNA to 28S rRNA can be transferred with similar efficiency from the same gel.

The procedure should be applicable for the detection of any RNA species for which an appropriate probe is available. We have successfully used as probes hybrid plasmids containing either segments of *D. melanogaster* chromosomal DNA or cDNA segments synthesized by reverse transcription of RNAs and also simian virus 40 DNA (data not shown). Since such probes can be labeled to high specific activities *in vitro* (13), the technique is extremely sensitive (Fig. 3*F*) and does not require *in vivo* labeling of the RNA.

The procedure is particularly suited to the detection of small amounts of specific RNA species present within complex mixtures of unrelated species. Examples of such uses might include studies of viral transcription, the detection of small quantities of specific mRNAs within cell extracts by probes derived from cloned DNA segments, and studies on precursor -product relationships of RNA species. We have found that specific mRNAs, which we estimate to comprise 1 to 5×10^{-4} of the total RNA in a cell extract, can be detected in a 5-μg sample within a few days of autoradiography, using probes with the specific radioactivities described here.

Berk and Sharp (21) have developed a different approach to the detection and sizing of small quantities of specific RNAs. In their procedure RNA is first hybridized in solution with the DNA probe; the specific DNA complement is then generated by digestion with the single-strand-specific nuclease S1 and sized by alkaline agarose gel electrophoresis. Their procedure therefore measures the length of regions in a given probe that are complementary to RNA species present in the mixture. By contrast, our DBM-paper transfer procedure measures the length of the RNA species itself; only a portion of that RNA

need be complementary to the probe. We note that the information generated by the two procedures may not be identical. For example, if a cloned DNA segment contained only a fragment of the region coding for a given RNA, then our procedure would measure the length of the RNA, while the procedure of Berk and Sharp would measure the length of that region in the cloned segment that was complementary to the RNA. If a given RNA species contained sequences complementary to several noncontiguous regions of a DNA probe, as is the case with cDm103 (15) and with adenovirus 2 (22), then again the two procedures would generate different information.

Preliminary results indicate that small denatured DNA molecules (<100 bases) can be effectively transferred from gels and coupled to DBM-paper. This extension of our procedure should overcome a limitation of the procedure of Southern (1) caused by the fact that small DNA fragments do not bind efficiently to nitrocellulose, and will be described in a subsequent paper.

Note Added in Proof. We have recently found that coupling is more efficient and DBM-paper more stable if potassium phosphate buffer, pH 6.5, is used instead of sodium borate, pH 8. After base treatment the gel is neutralized in 200 mM potasssium phosphate buffer, pH 6.5, and equilibrated and transferred in 25 mM potassium phosphate buffer, pH 6.5. Likewise, the final wash of DBM-paper before transfer is in 25 mM potassium phosphate buffer, pH 6.5. In addition, NBPC is now available from BDH Biochemicals Ltd., Poole, England (product no. 44168).

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