Nucleic Acid Hybridization with RNA Immobilized on Filter Paper

(carbonyldiimidazole coupling/*E. coli* RNA/fibroblast cell RNA/cellulose)

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ABSTRACT RNA has been immobilized in a manner suitable for use in molecular hybridization experiments with dissolved RNA or DNA by a nonaqueous solid-phase reaction with carbonyldiimidazole and RNA "dry coated" on cellulose or, preferably, on previously activated phosphocellulose filters. Immobilization of RNA does not appear to alter its chemical character or cause it to acquire affinity for unspecific RNA or DNA. The versatility and efficiency of this method make it potentially attractive for use in routine analytical or preparative hybridization experiments, among other applications.

We report here a technique in which RNA can be irreversibly immobilized on filter paper simply, conveniently, and efficiently with virtually any RNA species, and in which the RNA is available for the formation of a specific and stable hybrid structure.

Although immobilized DNA techniques for nucleic acid hybridization (1, 2) have been extensively used to measure nucleotide sequence homology between nucleic acid molecules, they are not suitable for RNA-RNA hybridizations or for RNA-DNA hybridizations that use radioactive DNA. Such hybridization experiments would be greatly facilitated by immobilization of RNA on a solid support. Several attempts have been made to do so (3-8), and the techniques have been useful in some circumstances.

In the method we present, RNA is covalently bonded to cellulose with the aid of the condensing agent carbonyldiimidazole (COIm₂) in an uninterrupted one-step reaction, or in a two-step reaction wherein the process is interrupted between the formation of an activated phosphate intermediate and its subsequent reaction with RNA. COIm₂ is neither toxic nor explosive, so that its application in these reactions requires no special apparatus and may proceed completely unattended. The simplicity, convenience, and versatility of this method recommends its use in routine or repetitive microanalytical scale experiments on filter discs, as well as in preparative-scale work with powder. In the two-step technique, the attachment yields with several RNA species were nearly quantitative and, in the one case tested—poly(A)—the RNA was over 90% available for hybridization to complementary nucleic acid. None of our RNA filters had any affinity for unspecific DNA or RNA.

MATERIALS AND METHODS

Materials. Pyridine and dimethylformamide (reagent grade, Matheson, Coleman and Bell) were dried by passage through a column of activated alumina and stored over Davison molecular sieve-4A (Grace Chemical Co., Baltimore, Md). Dicyclohexylcarbodiimide and 1-cyclohexyl-3-(2-mor-

pholinoethyl)carbodiimide metho-*p*-toluenesulfonate were obtained from Aldrich Chemical Co., Cedar Knolls, N.J., and carbonyldiimidazole was purchased from Sigma Chemical Co., St. Louis, Mo. Filter papers used in these studies were Whatman cellulose paper, grade 541 and Whatman phosphocellulose paper, grade P81. Polyuridylic acid, poly(U), and polyadenylic acid, poly(A), unlabeled and ³H-labeled, were purchased from Miles Laboratories, Kankakee, Ill.

Pulse-labeled *Escherichia coli* RNA was prepared as described (9). For the preparation of 28S ribosomal [³H]RNA, human fibroblast cells were labeled with [³H]uridine for 48 hr, then the total RNA was isolated by the hot-phenol technique (10). After treatment with electrophoretically pure deoxyribonuclease (Worthington Biochemicals, Freehold, N.J.), the 28S RNA was purified by polyacrylamide gel electrophoresis (11).

For attachment in the one-step reaction, these RNA preparations were converted to their tributylammonium salt by dialysis against 50 mM Bu₃N·HCl (pH 6) or by passage through a Biogel P2 column equilibrated with 5 mM Bu₃N·HCl (pH 6).

Preparation of RNA Filters (One-Step Reaction). RNA was spotted on 7-mm (diameter) paper discs air-dried for 10 min, then dried overnight under reduced pressure over P₂O₅ and KOH. During the subsequent bonding of RNA to filter paper, all operations were done in a dry atmosphere or in sealed vials. Organic solvent containing condensing agent (100 mg/ml, unless specific otherwise) was delivered to a vial containing replicate RNA-coated filters so that all of the filters were immersed. There is a slight but detectable transfer of RNA among filters, so loading of heterologous filters in the same vial was avoided. The bonding reaction was performed at the desired temperatures.

RNA filters were washed as follows: for single filters the condensing agent solution was removed by aspiration, replaced with a 1:1 mixture of formamide-1.5 mM NaCl-0.15 mM Na citrate (pH 7) and incubated at 35° for 1 hr. The solution was removed by aspiration and washed with NaCl-Na citrate by filtration. For batch treatment, filters were incubated in the formamide solution as described above, then in NaCl-Na citrate by repeatedly removing the solution by aspiration and replacing it with fresh solution. Finally, all filters were washed with acetone and dried with N₂. Either procedure removes 95% of the unreacted RNA.

Determination of filter radioactivity was influenced by the nature of the filter paper, the amount of RNA on the filter, and the nature of the processing treatment. An attempt to correct for this differential quenching was made by spotting known aliquots of RNA on appropriate filters.

Abbreviation: COIm₂, carbonyldiimidazole.

TABLE 1. Investigation of requirements for attachment of RNA to cellulose in a heterogeneous phase reaction

RNA	Solvent	Counter-ion	Filter	Condensing agent†	Input μg	Percent yield‡
Poly(A)	Pyridine	(Bu) ₃ NH ⁺	Cellulose	COIm ₂	4	82.0 ± 2.0
	Dimethylformamide	(Bu) ₃ NH ⁺	Cellulose	COIm ₂	4	68.0 ± 4.0
	Pyridine	K ⁺	Cellulose	COIm ₂	4	43.0 ± 3.0
	Pyridine	(Bu) ₃ NH ⁺	P-cellulose	COIm ₂	4	37.0 ± 1.0
	Pyridine	(Bu) ₃ NH ⁺	Cellulose	DCC	4	2.3 ± 0.1
	Pyridine	(Bu) ₃ NH ⁺	Cellulose	CMEC	4	<2
Pulse-labeled (<i>E. coli</i>)	Pyridine	(Bu) ₃ NH ⁺	Cellulose	COIm ₂	1,533	95.0 ± 0.1
	Dimethylformamide	(Bu) ₃ NH ⁺	Cellulose	COIm ₂	1,533	55.0 ± 2.0
	Pyridine	K ⁺	Cellulose	COIm ₂	644	64.0 ± 4.0
	Pyridine	(Bu) ₃ NH ⁺	P-cellulose	COIm ₂	1,533	64.0 ± 6.0
	Pyridine	(Bu) ₃ NH ⁺	Cellulose	CMEC	1,533	<2
	Pyridine	(Bu) ₃ NH ⁺	Cellulose	COIm ₂	1,200	50.0 ± 2.0
Fibroblast 28S	Pyridine	(Bu) ₃ NH ⁺	P-cellulose	COIm ₂	700	55.0 ± 3.0
4S Mouse*	Pyridine	K ⁺	Cellulose	COIm ₂	32,782	81.0 ± 6.0
[³ H]U-labeled	Pyridine	K ⁺	P-cellulose	COIm ₂	30,735	18.0 ± 1.0

Investigation of requirements for attachment of RNA to cellulose in heterogeneous phase reaction. Condensing agents were used at 100 mg/ml (w/v), and the reaction was performed for 8 hr at 37°.

* Cultured L-1210 mouse cells.

† CMEC, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluene sulfonate; DCC, dicyclohexylcarbodiimide.

‡ Percent yields were calculated as in *Methods*; average deviations for triplicate determinations are shown.

Preparation of Activated Phosphocellulose and RNA Filters (Two-Step Reaction). Phosphocellulose filter discs (7-mm diameter) were converted to the tributylammonium salt, then dried overnight under reduced pressure over P₂O₅ and KOH. COIm₂-dimethylformamide (100 mg/ml) was added to a vial containing these filters so that they were

immersed. The vial was sealed and allowed to stand overnight at room temperature. Excess COIm₂ was removed by washing three times with Me₂ formamide and three times with acetone. Finally, the filters were dried under a stream of dry nitrogen. The filters retained activity for at least 3 months when stored at -20° over a desiccant. RNA was attached by drying an aliquot of the RNA solution on an activated disc and allowing the reaction to proceed at 50° overnight in pyridine. Unreacted RNA was removed as described for the one-step reaction.

Hybridization with RNA Filters. Filters were immersed in 0.1 ml of a solution containing (final concentrations): 50% formamide, 0.45 M NaCl-0.045 M Na citrate, 0.01 M Tris (pH 7.2), and dissolved labeled nucleic acid (12). Incubation was routinely done at 36° for 24 hr in screw-cap vials. Filters were then washed with 0.45 M NaCl-0.045 M Na citrate by either of the procedures described above and assayed for radioactivity. Ribonuclease treatment was performed as described (2).

RESULTS

The one-step system

Our objective was to attach RNA covalently to filter paper by the formation of a bond between the 5'-terminal phosphate of the RNA and hydroxyl groups on the paper or, conversely, between ribosyl hydroxyl groups (or phosphate) of the RNA and the phosphates of phosphocellulose. Condensations of this type are most efficiently done in organic solvents, usually with carbodiimides (3) or, less frequently, with carbonyldiimidazole as a condensing agent (15).

Table 1 presents the results obtained when RNA preparations coated on cellulose or phosphocellulose filter discs were

FIG. 1. Influence of temperature, time, and concentration of COIm₂ on the bonding of poly(A) to cellulose. Cellulose filters were coated with 4 μg of [³H]poly(A) (Bu)₃NH⁺ and dried as in *Methods*. Duplicate filters were reacted in COIm₂-pyridine (100 mg/ml, w/v) for the appropriate times at 37° and 20° as shown, then washed and counted, and yields were calculated as in *Methods*, or reacted at 37° for 8 hr at various concentrations of COIm₂ in pyridine (*insert*). Upper curve, 37°. Lower curve, 25°.

reacted under various conditions in a one-step reaction. Although all combinations of variables were tested (solvent, paper, condensing agent, and counter-ion), only the best set of conditions and the effect of changing single variables are presented; the effects of changing a variable in the various combinations were always qualitatively similar to the changes seen in Table 1. When COIm₂ is used as condensing agent and pyridine as solvent, the attachment of various RNAs proceeded in good yield. All experiments with the carbodiimide condensing agents were unsuccessful in the solid-phase reaction. When the COIm₂-pyridine system was used, the best yields were obtained with cellulose paper and with the tributylammonium salt of the RNA. Results in the single-step reaction, although highly reproducible within any given set of experimental conditions, did vary (by about 20%) with different preparations of poly(A) (tributylammonium salt), but not with different preparations of *E. coli* pulse-labeled RNA.

Fig. 1 shows that the attachment reaction is dependent on the temperature and the duration of the reaction, and on the concentration of COIm₂. The yield of attached RNA in a 4-hr attachment reaction is also proportional to the amount of RNA dried on the filter (Fig. 2) until a value of fixed RNA of 4 μ g is reached—roughly 10% of the capacity of nitrocellulose filters for DNA from an equivalent surface area comparison. The plateau reached is a kinetic one, however, since 18 μ g can be attached in a 5-day reaction in a 62% yield (data not shown).

Poly(U) bonded to filter paper is stable to formamide-0.90 M NaCl-0.090 M Na₃ citrate (12) at 35° for at least 3 months. Filter-bound poly(U) and *E. coli* RNA are more than 90% sensitive to ribonuclease A, or are 100% sensitive to alkali digestions. When subsaturating amounts of [³H]-poly(U) were hybridized (formamide-NaCl-Na citrate solvent) (12) to immobilized poly(A) (4 μ g coated on cellulose, reacted 8 hr in COIm₂-pyridine), the hybrid was over 90% resistant to ribonuclease A and had a *T_m* of 62° in 0.3 M NaCl. It appears that the properties of polynucleotides, either attached to cellulose filters or in aqueous solution, are very similar (13). Hybridization experiments have also been done successfully with natural RNA immobilized on filter paper, and will be reported elsewhere.

Prolonged incubation of nucleosides under attachment conditions leads to irreversible nucleoside modification (W.C.S., unpublished data) but, if the reaction is performed for 4 hr or less, the modification is reversible by treatment with 50%-saturated ammonia at room temperature for 1 hr. This treatment does not remove *E. coli* RNA or poly(A)

Fig. 2. Capacity of cellulose filters as a function of poly(A) input. Cellulose filters were coated with [³H]poly(A) (Bu)₃NH⁺ and dried. Replicate filters were reacted in COIm₂-pyridine (100 mg/ml, w/v) for 4 hr at 37°, washed, and counted, and yields were calculated. Open circles and points denote separate experiments.

Fig. 3. Reaction of phosphates with carbonyldiimidazole. See text for explanation.

attached under the above conditions (see also ref. 14), and serves additionally to "deactivate" the surrounding paper.

"Mock" filters, lacking RNA but carried through the attachment schedule, do not interact significantly (0.1–1.0%) with dissolved labeled nucleic acid, when filters are washed as described (2). The nucleic acids tested include poly(U), poly(A), pulse-labeled RNA from *E. coli*, 28S ribosomal RNA from cultured human fibroblasts, 70S RNA from tumor viruses, DNA from cultured human fibroblast cells, and the DNA product synthesized by RNA-directed DNA polymerase from 70S tumor-virus RNA. This "noise" level is especially low when the filters are treated with ammonia, as described above. In our experience, *the only filters that can suitably measure noise levels are those that have been through the entire attachment schedule.*

The two-step system

In the one-step system, COIm₂ can react readily with atomatic amines (15), with the possible danger of modifying bases or of cross-linking bases to the cellulose through urethane linkages; therefore, *reaction conditions should be chosen that minimize the exposure of RNA to COIm₂.* To circumvent this problem, we explored a two-step system in which phosphocellulose was first converted to "activated" phosphoimidazolyl-cellulose in the absence of RNA.

The dependence of the one-step reaction on temperature and counter-ion is consistent with the chemistry of COIm₂ reactions with phosphoric acids (15, 16) and of phosphoimidazoles with alcohols (15, 17) in homogeneous solution, thus permitting us to predict optimal conditions in the heterogeneous phase system. Phosphate monoesters (I) react smoothly with COIm₂ (II) at room temperature to yield monoester-monoimidazolyl phosphinates (III) (Eq. 1, Fig. 3). This reaction shows no dependence on proton concentration (16), while the subsequent esterification of III by alcohols is strongly dependent on proton concentration and proceeds optimally at a higher temperature, around 50° (17) (Eq. 2, Fig. 3). Thus, the attachment of RNA to filter paper could be done in two separable steps, having discrete reaction optima. In addition, compounds of type III have two very useful properties; they are conditionally stable in water above pH 7 and their reaction with alcohols is faster than their reaction with amines (17).

Accordingly, phosphocellulose filters were first reacted with COIm₂ under mild conditions. Excess COIm₂ was then removed and the "activated paper" was stored over Drierite at -20°. RNA was attached simply by drying an aliquot of the RNA solution on an activated disc and reaction at an elevated temperature. In this system, the counter-ion is relatively unimportant and the attachment reaction is done

in the absence of free COIm₂. The RNA-paper is then "deactivated" with 50%-saturated ammonia for 2-10 min at room temperature, washed, dried, and stored at -20°.

Preliminary experiments with this approach have given results superior to the one-step reaction. All RNAs tested were attached in near-quantitative yields (80-100%); at least 90% of immobilized poly(A) could form a double-stranded hybrid structure with dissolved poly(U) (13).

DISCUSSION

The immobilized RNA method is well suited for either preparative or analytical purposes. As a preparative tool, it can aid in the rapid isolation of DNA regions or RNA molecules that are complementary to a given RNA species. While the isolation in pure form and characterization of redundant genetic loci, such as the genes coding for ribosomal RNA and tRNA, is easily within the scope of present technology, the practical isolation of unique genes is likely to constitute a more difficult problem. The identification, isolation, and characterization of the mRNA molecules in infected cells that are coded by genomic RNA of the RNA-containing viruses should be greatly facilitated by the immobilized RNA method. Additionally, the isolation of proteins that specifically interact with RNA molecules might also be feasible by the use of immobilized RNA.

The convenience and versatility of the technique, especially of the two-step variation, renders it particularly useful for analytical experiments. Recently, attention has become focused on homology between RNA molecules and DNA copies synthesized *in vitro* by RNA-directed DNA polymerase (18). The two methods used to assay hybrid formation are isopycnic centrifugation in Cs₂SO₄ (19) and nuclease resistance (20). The immobilized RNA method has the advantages of convenience and versatility over the Cs₂SO₄ technique, and of accuracy and sensitivity over the nuclease technique.

Finally, the method we have described should be easily applicable to DNA-DNA hybridizations. Though we have not explored it, the attachment reaction should be similar, if not the same, for DNA as for RNA (21). The relatively low noise levels we observe with radiolabeled DNA should encourage adaptation of the technique to the study of DNA-DNA interactions.

Though the immobilized RNA method has several advantages over existing techniques, it contains potential pitfalls that must be recognized. The attachment reaction may be capable of modifying bases; therefore, minimum attachment conditions (in terms of time and COIm₂ concentration) should be established for each system. Second, the rate of the hybridization reaction in our hands is unusually slow; on the order of 10-20% of the rate in homogeneous solution. The cause of this has not been determined, but likely causes include multiple attachment points between the RNA and the paper, nucleoside modification, or low diffusion rate in the paper matrix.

It should be noted that nuclease treatment of hybrids as an error detection technique for immobilized RNA is not as straightforward as in the case of immobilized DNA; in general, those nucleases that destroy the unreacted dissolved nucleic acid will tend to remove the immobilized RNA from the filter. In many cases, use can be made of the observation (22) that high temperature treatment destroys nuclease-sensitive complexes; in other cases, long DNA tails attached to a stable hybrid will preclude this alternative. When nu-

cleases are used, a reasonable approach is to first wash the filter-bound hybrid structure, then incubate it in a small volume with nuclease and to assay both the filter and the nuclease-containing fluid for the presence of hybrid structures. An obvious corollary to this discussion is that removal from the filter of hybrid regions by appropriate nucleases is a convenient method for preparing those hybrid regions.

In summary, the condensing agent COIm₂ can be used to immobilize virtually any RNA species in high yield to cellulose. Though the quality of hybrids formed with the COIm₂-immobilized RNA technique is comparable to similar hybrids formed in homogeneous solution, it should be noted that conditions for maximizing the rate of hybrid formation have not been realized. Inasmuch as the bonding of RNA to cellulose or phosphocellulose depends on the nature of the solvent, on the availability of protons, on the duration and temperature of the attachment reaction, and on the concentration of condensing agent, all of these parameters are amenable to manipulation towards achieving maximum hybrid yield. In addition, the simplicity, convenience, and versatility of this method make it potentially attractive for many other applications.

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