Nucleic Acid Hybridization Using DNA Covalently Coupled to Cellulose

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Summary

We describe a method for linking RNA and DNA covalently to finely divided cellulose through a diazotized aryl amine, which reacts primarily with guanine and uracil (thymine) residues of single strands. The high efficiency of coupling and high capacity of the cellulose for nucleic acid make possible a product with as much as 67 μg of nucleic acid per mg of cellulose. The product is especially suitable for hybridization experiments where very low backgrounds are important, and it is stable in 99% formamide at 80°C so that hybridized nucleic acid can be recovered easily. Full length linear Simian Virus 40 (SV40) DNA, produced by cleavage of SV40(I) DNA with S1 nuclease, can be coupled to diazo cellulose with an efficiency of 80–90%, and is effective in hybridization experiments with SV40 DNA, complementary RNA synthesized in vitro from SV40(I) DNA with E. coli RNA polymerase, and the SV40-specific fraction of total RNA from SV40-infected and transformed cells. In these experiments an excess of cellulose-bound DNA was used, and the efficiency of hybridization was about 90% when ribonuclease treatment of the hybrids was omitted.

Introduction

DNA–DNA or DNA–RNA hybrids formed in solution can be detected with a nuclease specific for single strands or isolated by chromatography on hydroxyapatite. (For example, with SV40 DNA, see Sambrook, Sharp, and Keller, 1972; Hansen, Pheiffer, and Hough, 1974.) DNA–DNA reannealing competes with DNA–RNA hybridization in solution, especially if the DNA is in excess. Although this difficulty can be eliminated by immobilizing the DNA on nitrocellulose filters (Gillespie and Spiegelman, 1965) or in agar (Bolton and McCarthy, 1962; Hansen et al., 1974), variable loss of DNA from the filter and high background levels in agar complicate the results. (For examples of the use of filters with SV40 DNA, see Westphal and Dulbecco, 1968; Haas, Vogt, and Dulbecco, 1972; Holzel and Sokol, 1974). Loss from the filter can be particularly significant in experiments designed to quantitate low levels of specific RNA within a larger heterogeneous pool, since any RNA which hybridizes to DNA in solution is not detected.

To circumvent these problems, we have developed a new method for hybridization using DNA covalently linked to cellulose. Shih and Martin (1974) coupled SV40 DNA to cellulose powder through the terminal phosphate groups using a water-soluble carbodiimide according to Gilham (1971). Poonian, Schlabach, and Weissbach (1971) and Arndt-Jovin et al. (1975) have coupled DNA to agarose activated with CNBr. These preparations have been used successfully in affinity chromatography, but their application to sensitive analytical hybridization may be limited because of the large amount of support material used. Residual positive charges on the agarose, generated as a consequence of CNBr activation, might also contribute to high background. The coupling procedure we describe is a modification of the one developed by Gurvich, Kuzovleva, and Tumanova (1961) for linking proteins covalently to finely divided aminobenzyloxymethyl cellulose. The high capacity of the cellulose and the facility with which the reaction can be performed are major advantages of the method. The DNA–cellulose can be used analytically to detect low levels of a specific nucleotide sequence within a larger heterogeneous pool as in filter hybridization techniques, or it can be used preparatively.

Results

Covalent Linkage of DNA to Cellulose

Single stranded DNA can be linked covalently to reprecipitated, finely divided m–aminobenzyloxymethyl cellulose after the primary aryl amino groups have been diazotized as described by Miles and Hales (1968). In the first experiment of Table 1, only 8% of the input SV40 DNA was coupled to the diazo cellulose in borate buffer under conditions used successfully by Miles and Hales (1968) for the coupling of proteins. The DNA in this experiment had been denatured at pH 12 immediately before the diazo cellulose was added, but was extensively renatured during coupling at high DNA concentration at 4°C (pH 8). However, when the DNA is kept denatured by doing the coupling in 70% or 80% dimethylsulfoxide (DMSO), more than 80% of the input can be added to the cellulose routinely. No DNA remains stably bound to amino cellulose which has not been diazotized. Experiments 2–4 of Table 1 show that the efficiency of the reaction depends on the concentration of diazo cellulose, but is relatively independent of the concentration of DNA. In experiments using from 20–800 μg of SV40 DNA at concentrations from 100–300 μg/ml, about 90% of the DNA can be linked covalently, provided that the cellulose concentration in the reaction mixture is at least 8–10 mg (dry weight)/ml. The percentage of DNA coupled decreases to 40–50% when the cellu-

Table 1. Coupling of DNA to Diazo Cellulose

Experiment	SV40 DNA	Buffer	DNA (μg/ml)	Diazo Cellulose (mg/ml)	% DNA Coupled	Temperature (°C)
1	Sonicated (6–7S)	Borate	245	12	8	4
		70% DMSO		9.5	87	
		70% DMSO		10	0	
				(NH$_2$–cellulose)		
2	S1 linears (16S)	70% DMSO	90	8	80	4
		80% DMSO	280	11	88	
3	S1 linears (16S)	80% DMSO	100	22	99	25
				2.2	43	
				0.5	4	
4	S1 linears (16S)	80% DMSO	200	2.2	45	25
				2.2	53	4

^{32}P-labeled SV40(I) DNA, sonicated to an average size of 6–7S or digested with S1 nuclease to full length linear size (16S), was suspended in 0.2 M borate buffer (pH 8), and diluted with additional buffer or DMSO. DNA in borate alone was denatured at pH 12, placed on ice, and readjusted to pH 8 immediately before addition to cellulose. DNA solutions were added to diazotized cellulose in small tubes, and the suspensions were stirred continually for 48 hr. The percentage of DNA coupled was determined from ^{32}P stably bound to the cellulose following extensive washing as described in the text.

lose concentration is reduced to 2 mg/ml, and the coupling efficiency is very poor if the cellulose concentration is reduced further. When E. coli DNA or salmon sperm DNA (200 μg/ml) are coupled to cellulose (10 mg/ml), about 60% of the DNA remains stably bound. This decrease in the percent of DNA which couples compared to SV40 DNA is not understood. E. coli tRNA (2 mg/ml) coupled to diazo cellulose (2 mg/ml) in 70% DMSO with 47% efficiency.

At cellulose concentrations above 8–10 mg/ml, the reaction is complete within 24 hr. At lower concentrations, 48 hr are required to achieve maximum coupling. The time course and extent of the coupling reaction are about the same at 4°C or 25°C.

The amount of DNA stably bound to the cellulose is determined after thorough washing with 80% DMSO and 0.1 × standard saline citrate (SSC, 0.15 M NaCl–0.015 M sodium citrate). When DNA denatured before addition of cellulose is coupled without DMSO, as much as 50% of the DNA initially bound can be removed by washing with 80% DMSO or 99% formamide. However, less than 10% of bound DNA is lost during such washing after reaction in DMSO, and most of this material is released in the low salt washes. The percentage of polynucleotide found linked covalently to the cellulose was the same whether determined using radioactively labeled DNA, by spectral analysis of acid hydrolysates of the DNA–cellulose, or by spectral analysis of the DNA which did not couple.

Table 2. Coupling of Nucleotide Homopolymers to Diazo Cellulose

	Polymer Concentration (A$_{260}$/ml)	% Coupled
SV40 DNA	5.65	87
Poly(U)	6.15	66
Poly(dT)	6.00	20
Poly(C)	6.25	<5
Poly(G)	5.65	41
Poly(A)	6.65	<5

Polymers were precipitated with ethanol and resuspended in 80% DMSO in 0.2 M borate buffer (pH 8). The molecular weights of all polymers were greater than 1×10^5, except poly(C) which was 3×10^4. Concentrations of the polymers were determined before the DMSO was added. Coupling was carried out at 4°C for 46 hr in a total volume of 0.2 ml at a cellulose concentration of 10 mg/ml.

The linkage between nucleic acids and the diazo cellulose was investigated using nucleotide homopolymers. The data of Table 2 suggest that coupling occurs best through guanine and uracil residues. Poly(dT) couples less well than poly(G) and poly(U), whereas poly(A) and poly(C) do not react with the diazo cellulose appreciably under the conditions tested. Although a low level of reaction with poly(A) and poly(C) cannot be excluded, the results do indicate that guanine and uracil (thymine) residues are probably the major sites of reaction.
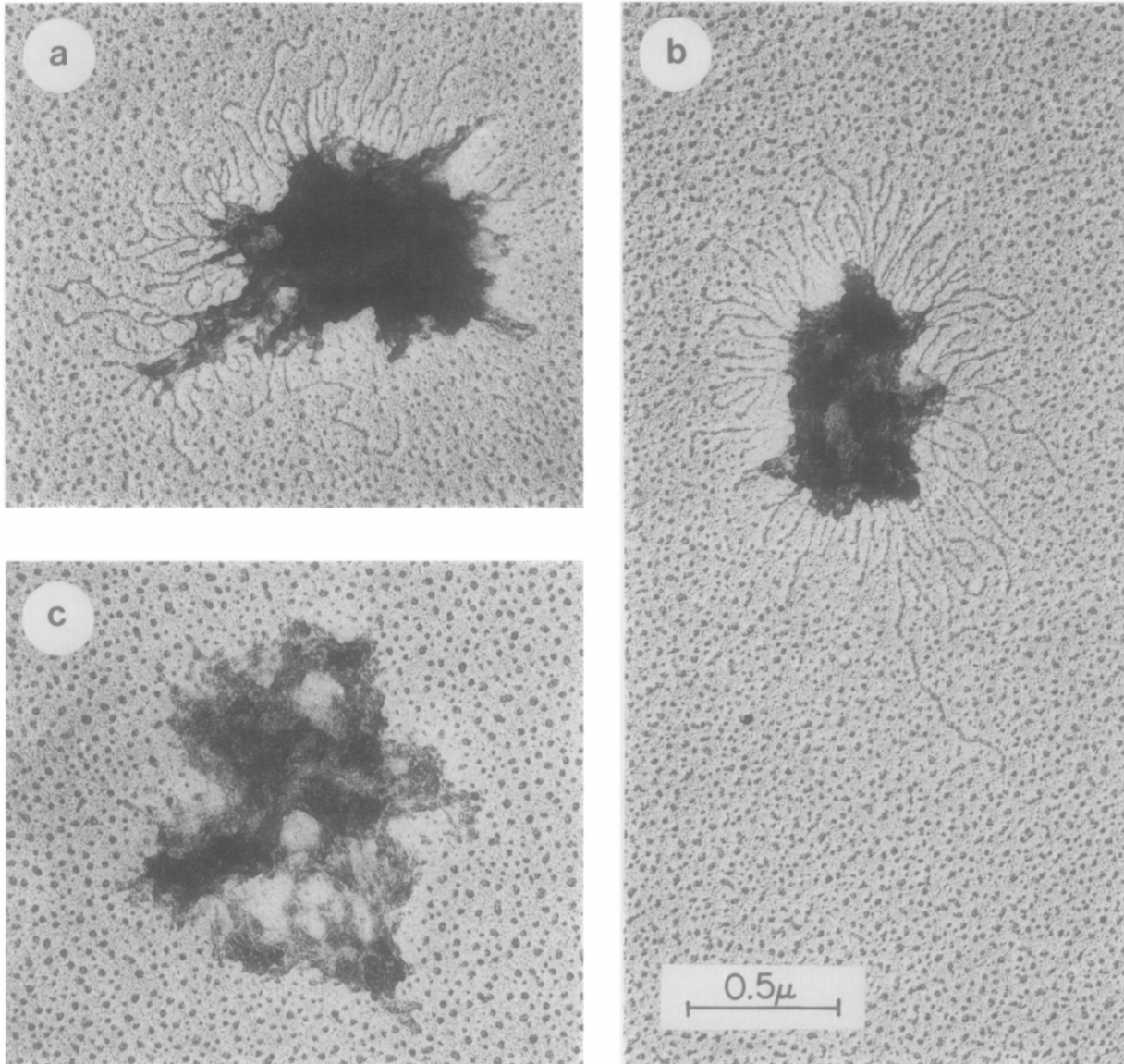
Figure 1. Electron Micrographs of DNA–Cellulose

Cellulose samples were spread in 50% formamide as described by Davis et al., (1971).
(a) SV40 DNA–cellulose, 22 μg DNA/mg.
(b) E. coli DNA–cellulose, 23 μg DNA/mg.
(c) Control cellulose diazotized and washed in the absence of DNA.

Accessibility for Hybridization of Coupled DNA

The availability of DNA linked to cellulose for molecular hybridization has been examined in several ways. Figure 1 shows electron micrographs of SV40 DNA–cellulose, E. coli DNA–cellulose, and control cellulose (subjected to the diazotization and washing procedures, but never exposed to DNA). Even though at least 8 equivalents of full length SV40 DNA are coupled to the cellulose particle in Figure 1a, there appears to be minimal self-annealing of the DNA as judged by comparison with the E. coli DNA–cellulose in Figure 1b, where the DNA must

be predominantly single stranded. It is probable that more DNA is actually present on the cellulose particles than is visible in the electron micrographs.

DNA–cellulose was tested for its ability to function in both DNA–DNA and DNA–RNA hybridizations. In 50% formamide buffer at 37°C for 24 hr, 96–99% of sonicated SV40 DNA (6–7S) hybridized selectively to SV40 DNA–cellulose at weight ratios of immobilized DNA:sonicated DNA of 24:1 or 48:1. RNA complementary to SV40 DNA (cRNA) was synthesized in vitro using E. coli RNA polymerase. As illustrated in Table 3, more than 70% of the cRNA hybrid-

ized selectively with SV40 DNA–cellulose, independent of the amount of DNA coupled per mg cellulose over an 11 fold range. Treatment with RNAase A caused a 15% decrease in the percentage of cRNA hybridized. This result is expected since sedimentation of the cRNA in 99% formamide and 0.1% sodium dodecyl sulfate (SDS) at 35°C indicated that about 12% of the preparation is larger than full length linear SV40 DNA. The relatively small effect of RNAase on the percentage of cRNA hybridized indicates that long regions of single stranded DNA must be available for hybridization.

An alternative method of examining the accessibility of the DNA is to hybridize increasing amounts of cRNA to a fixed amount of DNA–cellulose, as shown in Figure 2. At least 2.5 ng of cRNA (probably more at saturation) can be bound to 50 ng of DNA. Since most of the cRNA is complementary to only one of the two strands of SV40 DNA, at least one DNA strand in ten is accessible for hybridization with a long sequence of cRNA.

Efficiency of Hybridization of SV40 DNA–Cellulose with cRNA

The reaction between SV40 DNA–cellulose and cRNA was investigated more thoroughly to determine optimal standard conditions for assays of cell extracts containing SV40-specific RNA. At DNA concentrations between 1 and 120 μg/ml, 70–80% of the input cRNA hybridized reproducibly with the SV40 DNA–cellulose within 24 hr. Figure 3 shows kinetic data for a DNA concentration of 2.5 μg/ml and a cRNA concentration of 12 ng/ml. To evaluate the efficiency of hybridization reactions with cRNA

further, it was necessary to characterize the preparation used. Of 100 arbitrary units of cRNA, 17 were RNAase resistant. When incubated with SV40 DNA–cellulose under standard conditions for 24 hr, 75 units hybridized and 25 did not. Only 4
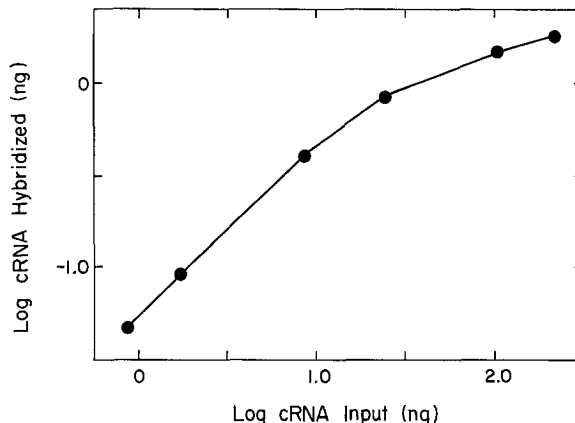

Figure 2. Hybridization of SV40 DNA–Cellulose with Increasing Amounts of cRNA

SV40 DNA coupled to cellulose (50 ng DNA, 22 μg DNA/mg) was hybridized with increasing amounts of cRNA in 50% formamide buffer for 48 hr at 37°C. The samples were washed, treated with RNAase (20 μg/ml, 1 hr, room temperature) in 2 × SSC and washed again. The cRNA hybridized was determined after elution with 99% formamide and 0.1% SDS as described in the text. Each point represents the average of duplicate determinations. Similar results were obtained when the RNAase treatment was omitted.
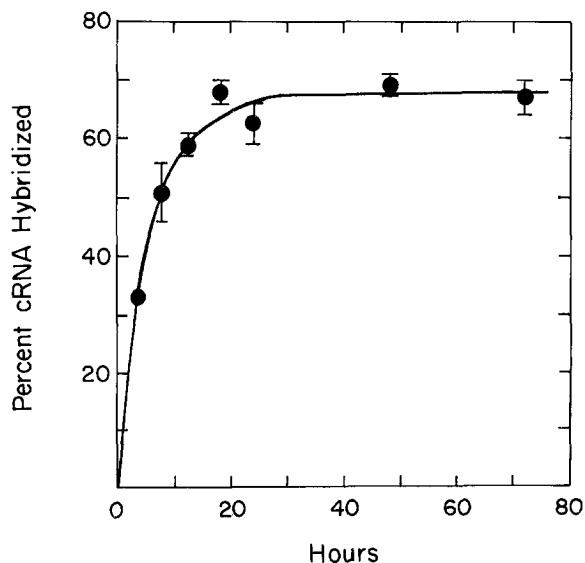

Figure 3. Kinetics of Hybridization of SV40 DNA–Cellulose with cRNA

SV40 DNA coupled to cellulose (500 ng DNA, 22 μg DNA/mg) was incubated with 2.5 ng cRNA in 50% formamide buffer at 37°C. The samples were washed, and the percentage of cRNA hybridized was determined after elution of the cRNA with 99% formamide and 0.1% SDS as described in the text. Each point represents the average of duplicate determinations. Similar results were obtained with samples treated with RNAase.

Table 3. Hybridization of SV40 cRNA with DNA Celluloses

DNA–Cellulose	μg DNA/mg Cellulose	% of Input cRNA Hybridized	
		–RNAase	+ RNAase
SV40	6	74.0	60.0
		76.7	60.3
	15	59.9	49.3
		70.4	49.9
	48	75.7	63.9
		74.7	66.9
	67	71.5	55.9
		70.8	55.7
Salmon Sperm	10	0.3	0.2
		0.5	0.3

DNA–cellulose containing 0.6 μg of DNA was incubated with SV40 cRNA (3.6 ng) in 50% formamide for 18 hr under standard conditions. The total amount of cellulose in each reaction mixture was normalized to 0.1 mg by adding carrier cellulose. Treatment with RNAase A (20 μg/ml) was for 1 hr at room temperature in 2 × SSC.

units of the cRNA which failed to hybridize in the first experiment hybridized to fresh SV40 DNA–cellulose in a second attempt. Therefore, 21 units of the original preparation failed to hybridize in two attempts. This material was completely RNAase sensitive, indicating that it was not double stranded; it probably represents transcripts of small amounts of cellular DNA contaminating the SV40 DNA preparation used as template. In addition to the 21 units of material not complementary to SV40 DNA, the cRNA contains 62 units of single stranded and 17 units of double stranded SV40 RNA. Since 75 units did hybridize in the first experiment, we conclude that the efficiency of hybridization was greater than 90%, and that in this case most of the double-stranded RNA did hybridize.

SV40-Specific RNA in Infected and Transformed Cells

To explore the utility of the technique for detecting low levels of SV40-specific RNA within the total RNA pool of eucaryotic cells, RNA was prepared from monkey cells (MA-134) productively infected by SV40 and from a hamster line transformed by SV40 (C13/SV28). MA-134 cells were harvested 72 hr after high multiplicity infection with SV40 and after labeling with ^{3}H–uridine for 20 min (71⅔–72 hr) or 24 hr (48–72 hr). RNA was extracted from the supernatant solution following lysis of the cells with SDS

in the presence of NaCl and removal of precipitated material by centrifugation (Hirt, 1967). Although RNA prepared in this way probably represents only about 50% of the total cellular RNA (Aloni, 1972), this procedure was chosen in order to compare our results with those of others. As indicated in Table 4, 10% of the RNA from cells pulse-labeled for 20 min with ^{3}H-uridine hybridizes specifically to SV40 DNA–cellulose. This agrees well with values of 10–20% and 10% reported by Aloni (1972) and Acheson et al. (1971) for viral-specific RNA prepared in the same way from SV40 and polyoma-infected cells and assayed using filter hybridization techniques. Table 4 also shows that about 0.7% of the RNA prepared from lytically infected cells labeled for 24 hr hybridizes with SV40 DNA–cellulose. This is within the range 0.1–1% reported by Khoury and Martin (1972) for the percentage of SV40-specific RNA in infected AGMK cells as measured by reassociation kinetics.

RNA from C13/SV28 cells was extracted after labeling with ^{3}H–uridine for 24 hr. As shown in Table 5, about 0.02% of the total labeled RNA hybridizes specifically to the SV40 DNA–cellulose. This value is 10 fold larger than the one reported by Sambrook et al. (1972) for SV40-specific RNA in SV3T3 cells (assayed by hydroxyapatite chromatography after hybridization in solution), but our result is within the range of the values 0.01–0.025% reported by

Table 4. SV40-Specific RNA in Infected Monkey Cells

Experiment	Labeling Period	μg DNA	μg RNA	% RNA Hybridized
1	20 min	10	2.3	8.0, 11.1
2	(71 2/3–72 hr)	10	1.9	9.8, 9.3
3		10	1.7	12.5
4		24	1.9	8.9
		24	0.95	13.2
				10.4 ± 1.8
5	24 hr	10	68	0.58, 0.67
	(48–72 hr)	10	143	0.74, 0.65
6		15	88	0.76, 0.83
		15	177	0.77, 0.73
7		20	34	0.91, 0.85
8		15	88	0.70, 0.73
				0.74 ± 0.09

RNA was extracted from MA-134 cells 72 hr after infection with SV40 and either 20 hr (71 2/3–72 hr) or 24 min (48–72 hr) after labeling with ^{3}H–uridine. The specific activity of RNA from cells labeled for 20 min was 6.4×10^3 cpm/μg, and from cells labeled for 24 hr, 2.6×10^4 cpm/μg. Hybridization with SV40 DNA–cellulose and E. coli DNA–cellulose was carried out in 50% formamide at 37°C for 40 hr as described in the text. RNAase treatment (20 μg/ml RNAase A and 1 μg/ml T1 RNAase) was for 1 hr at room temperature in 0.2 ml of 2 x SSC. Except for different RNA concentrations, which reflect differences in specific activity, the conditions in Experiments 1–4 were the same as those in Experiments 5–8. The percentage of input RNA hybridized was corrected for a background determined from hybridization with E. coli DNA–cellulose. For RNA labeled for 20 min, this background was 0.4% of the input, and for RNA labeled for 24 hr it was 0.009%. In Experiment 8, unlabeled total cellular RNA from uninfected MA-134 cells was used in place of yeast RNA as cold carrier in the hybridization mixture.

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