Isolation of Viral Specific RNA from SV40 Infected Cells by Viral DNA Chemically Linked to a Cellulose Matrix†

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ABSTRACT: SV40 DNA fragments chemically attached to neutral cellulose powder with a water-soluble carbodiimide have been used to isolate late lytic viral specific RNA from virus infected cells. Exhaustive hybridization to SV40 DNA reveals that virtually all of the isolated RNA molecules contain SV40 specific sequences. Comparison with SV40 cRNA prepared with purified *Escherichia coli* RNA polymerase and a SV40 DNA I template suggests that the purity of the isolated SV40 specific RNA is very close to 100%. The background level for the nonspecific binding of RNA to a purified cellulose matrix is very low. Retention of nonspecific RNA by SV40 DNA-cellulose is only 1.5% of the viral specific RNA isolated under saturating conditions for the column. Sedimentation in neutral sucrose suggests that the major 16S viral specific RNA has been isolated largely intact.

Isolation of distinct species of cellular RNA can be achieved on the basis of characteristic molecular size by methods such as sedimentation or electrophoresis. Since most of the mRNA molecules in eukaryotic cells appear to contain poly(A) sequences at their 3′-termini, they can also be selected by oligo(dT)-cellulose (Aviv and Leder, 1972) or poly(U)-Sepharose column chromatography (Lindberg et al., 1972). Using such methods, several RNA species have been isolated with considerable purity from cells in which a particular mRNA is the predominant constituent. In most cases, however, more selective techniques must be employed in order to obtain a unique species of RNA in a pure form. One approach involves the precipitation of polyribosomes with antibody prepared against a highly purified protein; the mRNA can subsequently be purified from the precipitated polyribosomes (Palmiter et al., 1972; Schechter, 1974). However, this method is applicable only in situations where a particular gene product is known and has been purified. In virus-infected or transformed cells, purification of virus-specific RNA can be achieved through hybridization with viral DNA. Preparative isolation of viral specific RNA would be of value for biochemical and biophysical characterizations of the RNA transcripts; such RNA species could also be used in cell-free translation systems to identify specific viral coded proteins. We have previously described a method for the stable covalent attachment of viral DNA fragments to a cellulose matrix which can be used for isolation of specific polynucleotide sequences (Shih and Martin, 1974). The coupling reaction involves activation of the terminal phosphate group of viral DNA fragments by a water-soluble carbodiimide, and its covalent attachment to neutral cellulose powder. In this paper, we report the application of SV40 DNA-cellulose column chromatography for the isolation of the SV40 specific RNA extracted from cells lytically infected by the virus.[1]

Materials and Methods

Virus Infection and Extraction of Cytoplasmic RNA. Cytoplasmic RNA was extracted from BSC-1 cells 48 h post-infection with SV40 at a multiplicity of 20–40 PFU/cell. These cultures were labeled with [5,6-^{3}H]uridine (100 μCi/ml, 41 Ci/mmol) from 36 to 48 h after infection for extraction of *late* lytic RNA. Cells were scraped from the monolayer, washed three times in phosphate-buffered saline, and resuspended in HeLa buffer (0.14 M NaCl–1.5 mM $MgCl_2$–0.01 M Tris (pH 7.5)). After addition of 0.05 volume of 10% NP40, cells were disrupted in a Dounce homogenizer. The cytoplasmic and nuclear RNAs were separated by centrifugation in an International centrifuge (2000 rpm, 5 min) and extracted with phenol–chloroform (50:50, v/v) at 25° after addition of 1% sodium dodecyl sulfate (Penman, 1966; Khoury et al., 1975). RNA was precipitated with two volumes of ethanol at −20°. The above steps for RNA extraction were performed at 4°. Uninfected cell RNA was prepared from Vero or 3T3 cells labeled with [^{3}H]uridine (100 μCi/ml, 41 Ci/mmol) or [^{14}C]uridine (0.5 μCi/ml, >50 mCi/mmol) for 12 h. The [^{3}H]uridine-labeled adenovirus 2 infected cell RNA was a gift from Dr. James Rose.

Preparation of SV40 DNA and cRNA. SV40 DNA was prepared from infected green monkey kidney cells as described elsewhere (Shih and Martin, 1974; Gelb et al., 1971; Khoury et al., 1975). SV40 cRNA was prepared in vitro using supercoiled SV40 DNA I template and *Escherichia coli* RNA polymerase (Shih and Martin, 1974). The cRNA was labeled with [^{3}H]UTP (5–8 mCi/μmol) and had a specific radioactivity of 2.3–3.5 × 10^6 cpm/μg. Purified *E. coli* RNA polymerase employed in these experiments contained negligible endogenous template activity (in absence of exogenous viral DNA only 0.1% of its full activity). Thus it is reasonable to assume that essentially all of the cRNA was transcribed from viral DNA template, and, therefore, SV40 cRNA was used as a reference in assessing the purity of SV40 specific RNA isolated from infected cells.

Affinity Chromatography for Purification of Viral Specific RNA. SV40 DNA fragments (about 300 000 daltons) (Gelb et al., 1971) were covalently attached to cellulose powder by 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide *p*-toluenesulfonate. The detailed coupling procedure was

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[1] Abbreviations used are: SV40, simian virus 40; SV40 cRNA, RNA synthesized in vitro with SV40 DNA I and *E. coli* RNA polymerase; EDTA, ethylenediaminetetraacetate; SSC, standard saline citrate (0.15 M NaCl–0.015 M sodium citrate, pH 7.0).

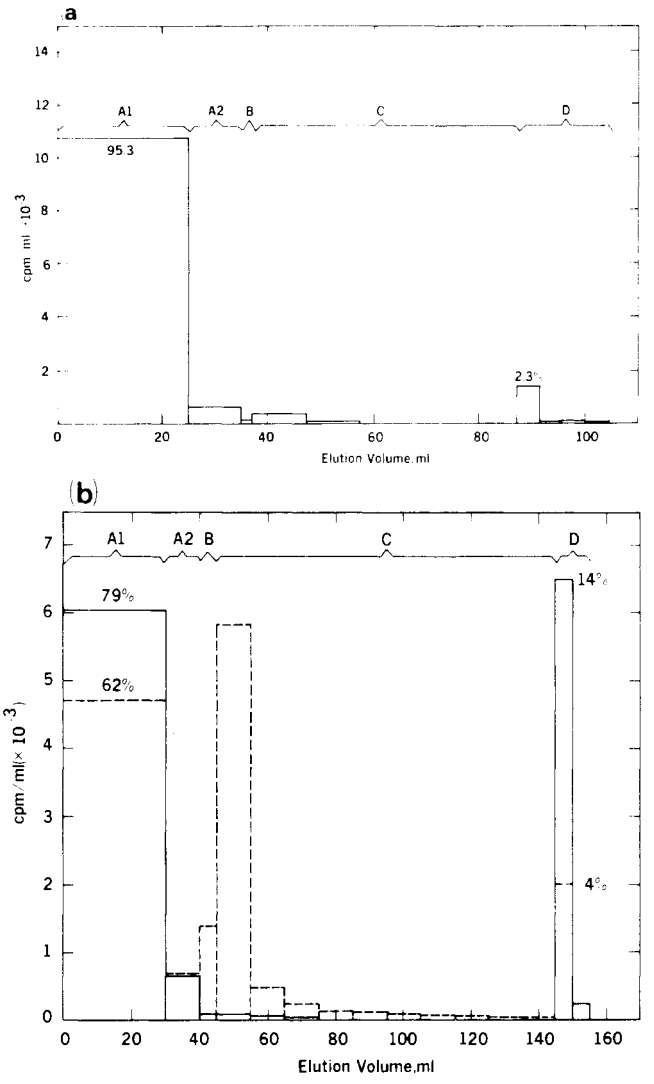

FIGURE 1: Affinity of [^{3}H]poly(A) for (a) SV40 DNA-cellulose and (b) untreated commercial cellulose. (a) [^{3}H]Poly(A) (305 000 cpm, 2.2 μg) in a circulant mixture of 30 ml was incubated with 170 mg of SV40 DNA-cellulose (ca. 17 μg of SV40 DNA) in a reassociation column of the continous hybridization system described in the text. After 69 h of incubation, the circulant was removed (A1). The column was successively washed with fresh circulant at 20° (A2); 0.3 M NaCl and 0.03 M sodium phosphate, pH 7.0 at 4° (B); and extensively with 0.015 M NaCl and 0.0015 M phosphate, pH 7.0 at 4° (C). Finally the retained material was eluted with deionized water at 60° (D). The total recovery of radioactivity in all fractions was 91% of the input. (b) [^{3}H]poly(A) (1.9 μg, 263 000 cpm) in a circulant mixture of 30 ml was incubated with 100 mg of commercial cellulose (Cellex-N-1, Bio-Rad) either in absence (—) or presence (- - -) of tenfold excess of poly(U) (18 μg).

described in a previous publication without significant alteration (Shih and Martin, 1974). The procedure is quite reproducible. Briefly, DNA in sodium 2-(*N*-morpholino)-ethanesulfonate buffer (pH 6.0) was mixed with an excess amount of carbodiimide. The mixture was applied in a dropwise fashion to a thin layer of highly purified cellulose powder. It was air-dried, and was periodically placed in a chamber saturated with water vapor. After the mixture stood overnight at room temperature, the cellulose powder was successively washed to remove noncoupled DNA with 0.05 M sodium phosphate (pH 7), 50% saturated NH_4OH solution, and 0.1 M sodium phosphate (pH 5 until reaching neutrality). The powder was then extensively washed with 50% formamide and finally with deionized water. The DNA-cellulose powder was dried and was stored at −20° in a desiccator until use. Cellulose powder (100 mg) containing about 10 μg of SV40 DNA was soaked overnight in 50% (v/v) formamide (pH 7), and heated at 50° for 3 min immediately prior to packing in a jacketed column (0.6 × 5 cm) at 4°. The cellulose column was then washed with 10 ml of hybridization medium containing 50% formamide (v/v), 1 mM EDTA, 0.2% dodecyl sulfate, 0.3 M NaCl, and 0.03 M sodium phosphate with final pH adjusted to 7.0. The RNA sample (2 mg to less than 1 μg) was dissolved in 7 ml of hybridization medium of the same composition and was circulated through the column with a pump at the flow rate of 0.1–0.2 ml/min at 4°. The entire apparatus was contained in a refrigerated chamber maintained at 4°. Hybridization was allowed to proceed by regulating the column temperature at 33° for 16–20 h. After incubation, the cellulose column was washed successively with 20 ml of fresh hybridization medium (omitting dodecyl sulfate), 10 ml of NaCl–PO_4 (0.3–0.03 M, pH 7.0), and 100 ml of dilute NaCl–PO_4 (15–1.5 mM, pH 7.0), all at 4°. RNA retained in the column was eluted with 1.5 ml of 95% formamide (pH 7) at 35°. After adjusting the final NaCl concentration to 0.15 M, RNA was precipitated by 2.5 volumes of ethanol at −20° overnight. The precipitate was pelleted by high-speed centrifugation (30 000 rpm in Spinco SW65 Ti rotor for 2 h). Finally, the RNA pellet was dissolved in a small volume of 0.01 M Tris (pH 7.0). The procedure followed was similar to that previously published (Shih and Martin, 1973, 1974).

Hybridization Methods for Analysis of Purified Viral RNA. Viral specific RNA sequences were detected and assayed by analytic hybridization to denatured SV40 DNA immobilized on nitrocellulose filters (Gillespie and Spiegelman, 1965). Each 6-mm diameter filter contained 1 μg of input SV40 DNA. Exhaustive hybridization to assess the purity of RNA was carried out in 0.25 ml of 50% formamide (v/v), 0.6 M NaCl, 2 mM EDTA, 0.2% dodecyl sulfate, and 0.06 M sodium phosphate with final pH of 7 at 37° for a total of 3 days involving three consecutive DNA filters. Prior to hybridization, filters were soaked for 30 min in fresh hybridization medium. Each filter was incubated with RNA for 20–25 hr, and was replaced with a new filter at the end of the incubation. The filters were immediately washed with 1 ml of 2 × SSC with gentle shaking overnight at 4°, dried, and counted in a toluene-based mixture. The background binding of the RNA to filters containing heterologous λ phage DNA was 0.1–0.5% of the input. In some experiments, filters were treated with pancreatic RNase A (15 μg/ml) in 2 × SSC. The exhaustive hybridization procedure was previously used by Lindberg et al. (1972). Acid-precipitable RNA does not decrease after 3 days of incubation under this condition. Alternatively, analytic hybridization was also performed in 0.25 ml of 1 M NaCl, 0.01 M Tris (pH 7.5), 2.5 mM EDTA, and 0.4% dodecyl sulfate for 16–20 h at 68°.

Sedimentation Analysis of Viral RNA. The size of RNA was analyzed by its sedimentation through a 10–20% sucrose gradient in 99% dimethyl sulfoxide (Strauss et al., 1968) or a 5–30% neutral sucrose gradient. [^{3}H]RNA and ^{14}C-labelled mammalian rRNA marker were counted with a liquid scintillation counter in toluene-based medium containing Triton X, and the spill into each channel was corrected by a computer program.

Other Materials and Methods. Fibrous cellulose powder, Cellex N-1, was obtained from Bio-Rad Laboratories,

Table I: Retention of Various RNA by SV40 DNA-Cellulose Column.[a]

RNA	Input		Retained		
	cpm	μg	cpm	μg[b]	% Input
1. [^{14}C]-3T3 RNA, cytoplasmic	5.18×10^6	492	405	0.038	0.008
2. [^{3}H] SV40 lytic RNA, cytoplasmic	19.3×10^6	110	109 000	0.63	0.57
3. [^{3}H] SV40 lytic RNA, cytoplasmic	67.1×10^6	1500	127 000	2.9	0.19
4. [^{3}H] SV40 lytic RNA, oligo(dT) selected	1.50×10^6	30	72 800	1.45	4.84
5. [^{3}H] SV40 cRNA	0.58×10^6	43	29 200	2.15	5.0

[a] Various RNAs in 10–30 ml were incubated with 100 mg of cellulose containing about 13 μg of SV40 DNA for 20 h. After incubation, the cellulose column was eluted by the standard procedure described in the text. [b] Amount of retained RNA was calculated by the specific radioactivity of input RNA. Nonuniform labeling of different RNA species is not corrected.

Richmond, Calif., and was further purified as previously described (Shih and Martin, 1974). [^{3}H]poly(A) was purchased from Miles Lab., Inc. (Elkhart, Ind.) (s_{20} of 7.8 as determined in 0.1 M NaCl–0.05 M PO_4 (pH 7.0)). The specific activity was 73 μCi/μmol of phosphate. Poly(U) and oligo(dT)-cellulose were purchased from P-L Biochemicals Inc., Milwaukee, Wis. Affinity chromatography involving oligo(dT)-cellulose column was performed as described by Aviv and Leder (1972).

Results

Evaluation of Binding Specificity. Poly(A) sequences appear to have a high affinity for many cellulose preparations (Kitos et al., 1972; Schutz et al., 1972). Since most animal virus and eukaryotic cellular mRNAs contain a poly(A) tract at the 3′ end (Brawerman, 1974), it is essential to prepare a cellulose matrix in which nonspecific binding is reduced to minimal levels. The high nonspecific binding of [^{3}H]poly(A) to commercial cellulose preparations (14%, Figure 1b) was resistant even to extensive washing with a dilute salt solution (15 mM NaCl–1.5 mM phosphate buffer). This affinity appears to be related to the single-stranded nature of the poly(A) sequences, since complexing them with a tenfold excess of poly(U) followed by washing with a dilute salt solution can lower the residual binding to 4% (Figure 1b). As it is seen in the elution profile, the effect of poly(U) complexing is mainly to loosen the affinity at low salt solution. When cellulose powder was purified by autoclaving in 1 M sodium bisulfite and extensively washed to remove any residual lignin impurities or minute crystalline cellulose (Shih and Martin, 1974), the level of poly(A) binding was reduced to about 2% (approximately 45 ng for the 170 mg of DNA-cellulose prepared from treated powder). SV40 DNA was linked to cellulose powder pretreated as described above for all subsequent experiments. To assess the specificity of binding of RNA to the SV40 DNA-cellulose column, a number of RNA preparations were tested. All cellular RNA was labeled for 12 h, a long labeling period, to allow incorporation of [^{3}H]uridine into mRNA and rRNA in relatively comparable proportions for each sample. The results summarized in Table I indicate that while only 0.038 μg (0.008% input) of the radiolabeled RNA from uninfected 3T3 (mouse) cells was retained by the column, 0.63 μg (0.57% input) of cytoplasmic RNA extracted from lytically infected BSC-1 cells was retained under similar conditions. At column saturation, 2.9 μg (0.19% input) of RNA was retained from 1.5 mg of late lytic SV40 cytoplasmic RNA. However, when RNA was first selected on oligo(dT)-cellulose columns, a much larger percentage of RNA (1.45 μg = 4.84% of input RNA) was bound to the DNA-cellulose column. To further test the saturating capacity of the columns used in these experiments, an excess of SV40 cRNA (all of which should be virus specific) was allowed to react with DNA-cellulose. Of the 43 μg of input cRNA, 2.15 μg (5%) was retained. It can be concluded from Table I, that at column saturation, the percentage of nonspecific binding of RNA to DNA-cellulose can be reduced to very low levels (less than 2% of the bound viral-specific RNA).

Isolation of Late Lytic SV40 Specific RNA and Assessment of Its Purity. Cytoplasmic RNA was extracted from BSC-1 cells 48 h post-infection (p.i.) with SV40. The RNA which had been labeled from 36 to 48 h p.i. with [^{3}H]uridine was subsequently purified by SV40 DNA-cellulose chromatography as described in Materials and Methods. The presence of SV40-specific sequences in various RNA preparations was assessed in a number of filter hybridization studies (Table II). In control experiments, less than 0.01% of the total counts of uninfected Vero cell RNA or adenovirus 2 infected KB cell RNA bound to SV40 DNA containing filters; similarly, there was negligible binding of any of the ^{3}H-labeled RNAs to control filters containing λ phage DNA. While a small but significant fraction (1.6–1.8%) of nonselected late lytic SV40 cytoplasmic RNA bound to the SV40 DNA containing filters, a considerably larger fraction of SV40 DNA-cellulose selected (19–35%) or SV40 cRNA (50–62%) bound to SV40 DNA filters under similar experimental conditions. A comparison of the binding of nonselected and DNA-cellulose column selected SV40 cytoplasmic RNA to poly(U) containing filters (2.8 vs. 48%) indicates that selected RNA has been enriched for poly(A) sequences and thus for mRNA. While the results of these experiments show clearly that SV40 DNA-cellulose column chromatography can provide a significant enrichment of SV40 RNA sequences, they do not allow a quantitative determination of the purity of the selected RNA.

In an attempt to determine the purity of the SV40 DNA column selected RNA, we employed exhaustive filter hybridization in 50% formamide at 37°. Three SV40 DNA filters were used consecutively, each for 19–24 h for each RNA sample. While minimizing the loss of DNA or DNA–RNA hybrids from the filters, and degradation of RNA,

Table II: Hybridization of [^{3}H]RNAs to Filters Containing SV40 DNA, Control DNA, or Poly(uridylic acid).[a]

RNA	Total RNA		RNA Bound to Filters Containing				
			SV40 DNA		λ DNA		Poly(U)
	cpm × 10^{-3}	μg	cpm	% Total	cpm	% Total	% Total
1. Vero cell	1900	450	112	<0.01	80	<0.01	
2. Ad 2 infected cell	612	12	44	<0.01	15	<0.01	
3. SV40 cRNA	5.9	0.035	3660	62	2	0.03	
	12.8	0.076	6400	50	5	0.04	
4. SV40 infected cell, cytoplasmic	254	3.8	4170	1.6	11	<0.01	2.8
	79	1.2	1410	1.8	0	0	
5. SV40 DNA-cellulose selected lytic RNA	5.0	0.11	1755	35	0	0	48.0
	27.4	0.57	5169	19	2	<0.01	

[a] Various ^{3}H-labeled RNAs were incubated with SV40 or control DNA filters (1 μg of input denatured DNA per filter) in 0.25 ml of solution containing 1 M NaCl, 0.04 M TES buffer (pH 7.5), 0.0025 M EDTA, and 0.4% sodium dodecyl sulfate for 16 h at 68°. Filters were then incubated for 1 h at 20° in 1 ml of standard saline citrate containing 10 μg/ml of pancreatic RNase, washed, dried, and counted in a toluene-based scintillation fluid. RNA remaining in the hybridization mixture was precipitated by 10% Cl_3CCOOH and was counted as above. Percentage of the total recovered Cl_3CCOOH precipitated RNA which hybridized to DNA filters was presented in this table. Cytoplasmic RNA extracted from cells 48 h after infection with SV40 was tested both before and after selection on SV40 DNA-cellulose columns for the percent binding to poly(U) containing filters as has been described by Sheldon et al. (1972).

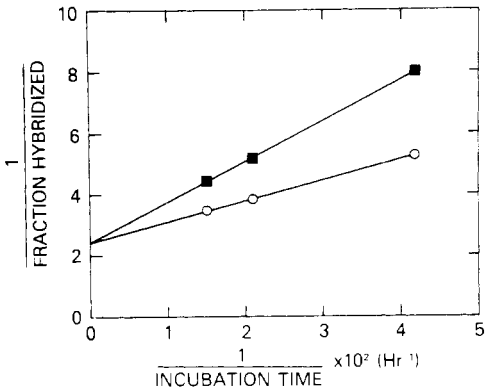

FIGURE 2: Comparison of hybridization to SV40 DNA filters of purified late lytic RNA and SV40 cRNA. Purified late lytic RNA (■) and SV40 cRNA (○) in 0.25 ml of 50% formamide, 0.6 M NaCl, 2 mM EDTA, 0.2% dodecyl sulfate, and 0.06 M sodium phosphate (pH 7.0) were each incubated consecutively with a set of three SV40 DNA filters. After incubation, the filters were treated with 15 μg of pancreatic RNase A in 1 ml of SSC for 60 min at 25°. Filters were washed, dried, and counted. Input purified lytic RNA was 9386 cpm (0.195 μg), and that of cRNA was 54 224 cpm (0.023 μg). Cl_3CCOOH precipitable radioactivity of input controls carried out in separate vials did not decrease at the end of incubation. 6-mm diameter SV40 DNA filters were cut from the same 5-cm diameter filters.

this technique allows an extrapolation to infinite incubation times. After RNase treatment, filters were counted and the double reciprocal plots of the cumulative fraction of [^{3}H]RNA retained on filters vs. the times of incubation are presented in Figure 2. Several observations can be made from this graph. First, the apparent rate (i.e., the fraction rate *not* the rate of amount of RNA hybridized) of cRNA hybridization is much faster (a greater slope) than that of isolated lytic RNA, perhaps related to the greater specific radioactivity (lower RNA concentration) for cRNA than for the lytic RNA. The extrapolation of both lines to infinite incubation times ($1/t = 0$) is coincident, suggesting that the DNA column selected lytic RNA and SV40 cRNA have similar purities. The intercept for both hybridization reactions, however, is 2.5, rather than 1.0, which is equivalent to a maximum binding of 40% rather than 100%. Since the purity of SV40 cRNA is likely to be closer to 100 than to 40%, the values obtained in this experiment are probably underestimates. A possible explanation for the reduction in maximum hybridization levels is the RNase sensitivity of some SV40-specific RNA. Thus weakly bound virus-specific RNA or tails of hybrid molecules must be degraded by RNase under the experimental conditions. Analysis of exhaustive hybridization data extrapolated to infinite times of incubations without RNase treatment (see Figure 3 and Table III) indicates that virtually all of the cRNA and DNA-cellulose column selected lytic SV40 RNA sequences are SV40 specific (the intercept for 1/fraction bound = 1.0, see Figure 3b). These values are not the result of adventitious binding of RNase susceptible RNA to filters, since the level of binding to control filters in the absence of RNase treatment is quite low (0.46 and 0.1%, Table III). In a comparable analysis (Figure 3a), about 17% of the oligo(dT)-cellulose selected RNA contained SV40 specific sequences. In these experiments (Table III and Figure 3), it can be seen that the concentration of SV40 specific RNA is related to the "apparent rate" of hybridization, i.e., DNA-cellulose selected RNA < oligo(dT) and DNA-cellulose selected RNA < SV40 cRNA.

The results in Figure 3 suggest that most, if not all, SV40 DNA-cellulose column selected molecules contain SV40-specific RNA sequences by virtue of their binding to the SV40 DNA filters. It is possible, however, that a fraction of nonviral sequences covalently linked to or associated with SV40 sequences might be present. Nevertheless, it can be concluded that DNA-cellulose chromatography provides a very high degree of purity of SV40-specific RNA molecules (close to 100%). This result is consistent with low level of background binding for the DNA-cellulose column (Table I).

Size of Isolated Virus-Specific RNA. For a number of analytical and functional studies it is important that the isolated SV40-specific RNA remain intact. Thus, we investigated the effect of DNA-cellulose chromatography on the size of selected RNA. Initial studies were performed with [^{3}H]SV40 cRNA, since all of the sequences have the potential to bind to the column, and therefore an analysis can be made both before and after chromatography. SV40 cRNA obtained in the excluded volume of Sephadex G-100 filtra-

Table III: Exhaustive Hybridization of Purified RNA to SV40 DNA Filters.[a]

RNA	Input		% Input Bound to			% Input not Bound
	cpm	μg	1st Filter	2nd Filter	3rd Filter	
(1) Late lytic RNA, oligo(dT)-cellulose selected	9 028	0.28	3.2 (1.32)	3.8 (2.7)	1.07 (0.82)	78
(2) Late lytic RNA, SV40 DNA-cellulose selected	5 173	0.16	28.2 (15.8)	17.4 (7.84)	3.2 (1.32)	51.2
(3) Late lytic RNA, oligo(dT)- and SV40 DNA-cellulose selected	1 914	0.06	43.4 (25.0)	15.1 (7.37)	9.3 (3.89)	39.2
(4) SV40 cRNA	16 139	0.007	56.1 (36.0)	23.6 (11.4)	5.4 (4.1)	22.1
Control experiments using λ DNA						
(5) Late lytic RNA, SV40 DNA-cellulose selected	9 386	0.19	0.46 (0.06)			
(6) SV40 cRNA	15 000	0.007	0.10 (0.04)			

[a] RNAs were incubated with SV40 DNA filters for the following period of time: first filter, 26 h; second filter, 23 h; and third filter, 24 h. Hybridization was carried out at 37° in 0.25 ml of 50% formamide (v/v), 0.6 M NaCl, 2 mM EDTA, 0.2% dodecyl sulfate, and 0.06 M sodium phosphate (pH 7.0). Parallel controls of input RNA were carried out in similar hybridization medium in separate vials containing no filter. No degradation of RNA was observed under these conditions. Filters were treated and counted as described in Materials and Methods. Control filters in experiments 5 and 6 were prepared with 1 μg of input λ phage DNA in each filter. After counting in a toluene-based scintillation fluid, filters were washed with toluene and were treated with RNase (10 μg/ml) in 2 × SSC for 60 min (25°). Values obtained were presented in parentheses.

tion, and further selected by neutral sucrose gradient sedimentation was analyzed in a Me_2SO-sucrose gradient (Figure 4a). A sample of this cRNA was mixed with 7 ml of hybridization medium, and was isolated by SV40 DNA-cellulose as described in Materials and Methods. Following 16 h of incubation, 28% of cRNA was retained by the column. The sedimentation profile of the isolated cRNA is shown in Figure 4b. Although quite broad, the center of the peak of the [^{3}H]cRNA following isolation, 14.4 S, is similar to that of the original cRNA (Figure 4a). In another experiment, cRNA was isolated following 66 h of incubation at 33°; still no appreciable change was observed as compared with the original RNA of 11 S (data not shown). Nevertheless, an incubation time of 16–24 h was routinely used for preserving the integrity of RNA molecules. The small amounts of fast sedimenting material seen at the bottom of the gradients in Figure 4b as well as Figure 5b are absent from the original RNA profiles. This material was observed only in freshly dissolved RNA precipitates, and was not found in column-isolated RNA sedimented without prior precipitation.

The sedimentation behavior in neutral sucrose gradient of the SV40 specific RNA purified from cytoplasmic fraction of BSC-1 cells late in lytic infection is shown in Figure 5 Panel (a) presents the sedimentation profile of ^{3}H-labeled oligo(dT)-cellulose selected total poly(A)$^+$ cytoplasmic RNA, cosedimenting with a ^{14}C-labeled rRNA marker. The sedimentation profiles of the isolated RNA preparations used in the hybridization studies of Figure 3 are seen in panel (b). The major peak of virus-specific RNA [with or without preselection on oligo(dT)-cellulose] appears at fraction 34 ($s_{20,w}$ value of 16 S) and probably corresponds to the more abundant of the two late lytic SV40 RNA species (16 S and 19 S) described by others (Weinberg et al., 1972, 1974; May et al., 1973).

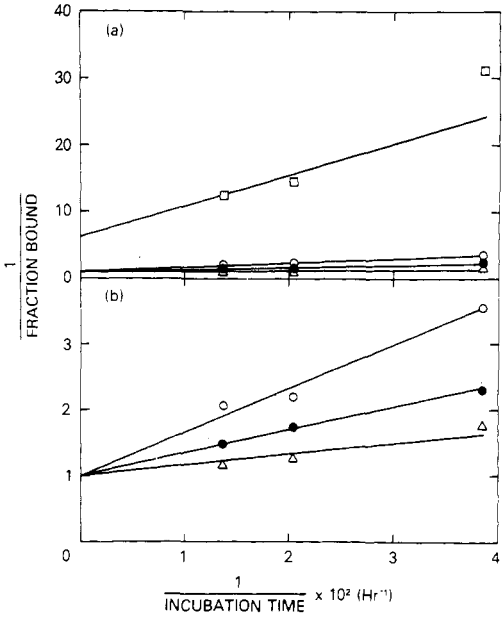

FIGURE 3: Exhaustive hybridization of various purified RNA to SV40 DNA filters. Cumulative fractions of RNA bound to SV40 DNA filters after various periods of incubation were presented in the double reciprocal plots. Data were taken from those in Table III. Radioactivity on the filters was counted after washing but without RNase treatment. (□) Oligo(dT)-cellulose selected late lytic RNA; (○) SV40 DNA-cellulose selected late lytic RNA; (●) oligo(dT)- and SV40 DNA-cellulose selected late lytic RNA; (△) SV40 cRNA. Panels a and b differ in scale.

Discussion

DNA-cellulose column chromatography offers a method for the selection of SV40 mRNA sequences which constitute about 1% of the total infected cell cytoplasmic RNA. Isolation of specific viral RNAs has previously been performed with viral DNA immobilized on nitrocellulose filters (Weinberg et al., 1972; Buttner et al., 1974), or in solution hybridization with viral DNA (Eron and Westphal, 1974). The present method offers the advantages of minimizing contamination by viral DNA and ensuring a relatively high purity of isolated RNA due to its low background. The cellulose matrix provides stability for hybrid-

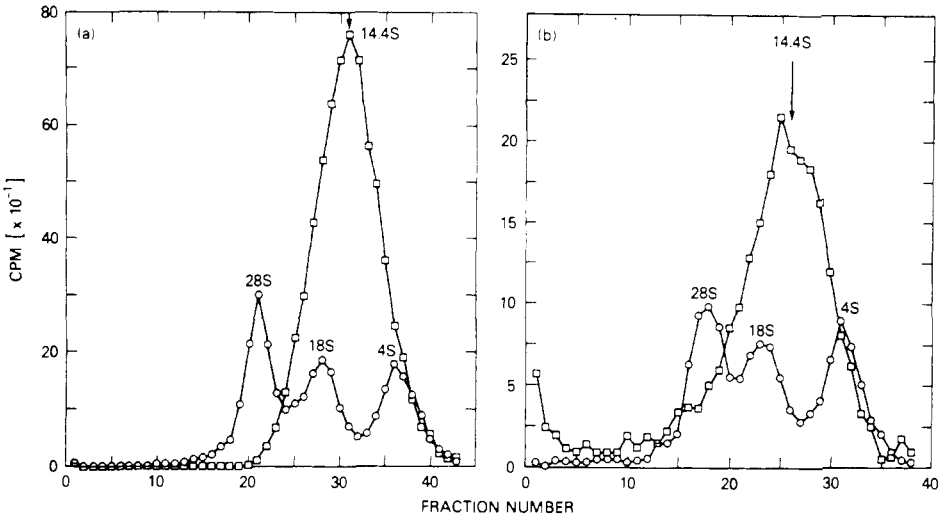

FIGURE 4: Sedimentation profile of [^{3}H]SV40 cRNA isolated by SV40 DNA-cellulose column as compared with that of the original RNA in dimethyl sulfoxide-sucrose gradient centrifugation. (a) 100 μl of the original [^{3}H]SV40 cRNA (11 000 cpm, 3 ng) in 0.01 M Tris (pH 7.5) and 1 μl of ^{14}C-labeled ribosomal RNA marker were mixed with 100 μl of Me_2SO and 20 μl of *N,N*-dimethylformamide. The mixture was layered on a 10–20% sucrose gradient in 99% Me_2SO (10 mM LiCl–1 mM EDTA, (pH 7.0)) in a 5-ml tube. It was centrifuged with a Spinco SW50.1 rotor at 48 000 rpm for 16 h (20°). (b) 100 μl of [^{3}H]SV40 cRNA isolated by SV40 DNA-cellulose column chromatography (4000 cpm) was sedimentated as in (a).

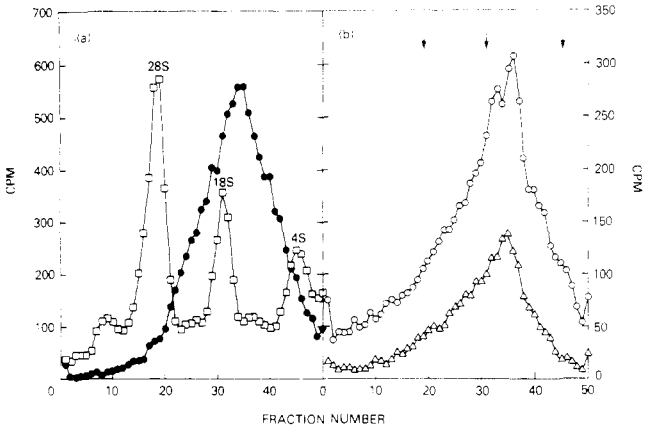

FIGURE 5: Sedimentation profiles of late lytic RNA isolated by oligo(dT) or SV40 DNA-cellulose. In panel a, late lytic RNA isolated by oligo(dT)-cellulose (●) (13 000 cpm) was mixed with [^{14}C]rRNA marker (□), and sedimentated through a 5–30% neutral sucrose gradient (0.1 M NaCl) in a Spinco SW 41 rotor at 33 000 rpm for 9 h (10°). In parallel tubes were RNA samples further purified by SV40 DNA-cellulose (△) (7000 cpm) and RNA isolated by SV40 DNA-cellulose alone (○) (14 800 cpm).

ization in various organic solvents for prolonged incubation times. Because of the stable linkage of DNA to the matrix, the DNA-cellulose can be reused several times. Appropriate preparation of the cellulose prior to use appears to be important in eliminating general binding of poly(A)-containing RNA sequences by virtue of their affinity for certain cellulose impurities.

The sedimentation behavior of the purified RNA in a sucrose density gradient suggests that the integrity of the isolated RNA molecules is good. The RNA eluted from specific DNA-cellulose columns can be used for a number of biochemical and biophysical studies including a determination of the primary sequence, terminal structures, and associated cellular elements. In addition virus-specific mRNA can be used to program cell-free translation system in an attempt to identify viral-coded functions; by selecting mRNA from cellulose columns which contain restricted sets of DNA sequences, one should be able to map specific proteins within the viral genome.

Purification of mRNAs by DNA-cellulose column chromatography will allow the characterization of small quantities of specific transcripts present in very low concentrations by minimizing the levels of "background" contaminating RNA. In providing an alternative to the general method of poly(A) selection for the isolation of specific mRNAs, this technique will permit one to study poly(A)$^-$ (Milcarek et al., 1974) as well as poly(A)$^+$ RNAs in eukaryotic cells and perhaps to define the relationship between the two species.

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References

Aviv, H., and Leder, P. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 1408–1412.

Brawerman, G. (1974), *Annu. Rev. Biochem. 43,* 621–642.

Buttner, W., Veres-Molnar, Z., and Green, M. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 2951–2955.

Eron, L., and Westphal, H. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 3385–3389.

Gelb, L. D., Kohne, D. E., and Martin, M. A. (1971), *J. Mol. Biol. 57,* 129–145.

Gillespie, D., and Spiegelman, S. (1965), *J. Mol. Biol. 12,* 829–842.

Khoury, G., Howley, P., Nathans, D., and Martin, M. A. (1975), *J. Virol. 15,* 433–437.

Kitos, P. A., Saxon, G., and Amos, H. (1972), *Biochem. Biophys. Res. Commun. 47,* 1426–1437.

Lindberg, U., Persson, T., and Philipson, L. (1972), *J. Virol. 10,* 909–919.

May, E., May, P., and Weil, R. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 1654–1658.

Milcarek, C., Price, R., and Penman, S. (1974), *Cell 3,* 1–10.

Palmiter, R. D., Palacios, R., and Schimke, R. T. (1972), *J. Biol. Chem. 247,* 3296–3304.
Penman, S. (1966), *J. Mol. Biol. 17,* 117–130.
Schechter, I. (1974), *Biochemistry 13,* 1875–1885.
Schutz, G., Beato, M., and Feigelson, P. (1972), *Biochem. Biophys. Res. Commun. 49,* 680–689.
Sheldon, R., Jurale, C., and Kates, J. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 417–421.
Shih, T. Y., and Martin, M. A. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 1697–1700.
Shih, T. Y., and Martin, M. A. (1974), *Biochemistry 13,* 3411–3418.
Strauss, J. H., Jr., Kelly, R. B., and Sinsheimer, R. L. (1968), *Biopolymers 6,* 793–807.
Weinberg, R. A., Warnaar, S. O., and Winocour, E. (1972), *J. Virol. 10,* 193–201.
Weinberg, R. A., Ben-Ishai, Z., and Newbold, J. E. (1974), *J. Virol. 13,* 1263–1273.

The Affinity Labeling of Amino Acids in or about the Active Center of DNA-Dependent DNA Polymerase I†

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ABSTRACT: The use of an affinity label and an inhibitor that shows relative specificity for one amino acid has led to the identification of two amino acid residues in or near the active center of DNA-dependent DNA polymerase I. [^{35}S]-β-D-Ribosyl-6-methylthiopurine periodate oxidation product ([^{35}S]MMPR-OP) and [^{14}C]phenylglyoxal ([^{14}C]PG) were used to elucidate the presence of a single lysine and arginine in or about the active center of the enzyme.

Since the earliest description by Kornberg et al. (1956a,b) and Bessman et al. (1957) of enzyme extracts from *Escherichia coli* that catalyzed the incorporation of deoxyribonucleotides into DNA, much work has been done to elucidate the structure and mechanism of action of the enzyme. These investigators, together with Richardson et al. (1964) and Jovin et al. (1969a), using further sophisticated purification techniques, demonstrated the requirements and structure of the Kornberg DNA polymerase I. In addition to polymerization, this enzyme possesses various other catalytic activities which have been described in excellent reviews by Kornberg (1969) and Goulian (1971). Recently, the possible mechanism of action of DNA polymerase I as a zinc metalloenzyme was reviewed (Mildvan, 1974). DNA polymerase I has recently been shown to function in DNA replication along with DNA polymerase II and III (Tait and Smith, 1974). However, even though the catalytic activities and functions are known, the exact enzymatic mechanisms involved in mediating catalysis are still open to investigation. To that eventual end, this communication describes the identification of two amino acid residues found in the active center of the Kornberg enzyme which demonstrate a role primarily in polymerization.

The role of lysine was determined by using an affinity label, the oxidation product of β-D-ribosyl-6-methylthiopurine (MMPR-OP).[1] This compound was found by Kimball et al. (1968) to be an inhibitor of DNA polymerase of the Ehrlich ascites tumor. This was one of the earlier works utilizing a compound that alters the catalytic mechanism of DNA polymerase by binding to specific sites on the enzyme. Spoor et al. (1970), using cell-free extracts of this tumor line, also found this compound to inhibit the activity of RNA polymerase. In addition, they showed that MMPR-OP would inhibit and affinity label RNase A (Spoor et al., 1973). Nixon et al. (1972) also found that the compound would affinity label the β subunit of RNA polymerase. Specifically, it was shown that there was the formation of a Schiff base between one of the aldehyde moieties of MMPR-OP and the ϵ-amino group of a catalytically active lysine residue in the initiation subsite of the enzyme. Wu and Wu (1974) have modified the MMPR-OP to produce a fluorescent compound that also affinity labels the β subunit of RNA polymerase. Since the above enzymes are enzymes of nucleic acid metabolism, this possible effect of MMPR-OP on DNA polymerase I was used to determine if the same affinity labeling action would apply to this enzyme.

Takahashi (1968) was able to inactivate RNase A some 80–90% with phenylglyoxal (PG) under mild conditions; pH 7–8 and 25 °C. The mechanism of inhibition involved the condensation of two phenylglyoxal molecules with the guanidino group of arginine. The arginine residues of RNase A affected were Arg-39 and Arg-85 which were shown to be those closest to the active center. In addition, experimental conditions were employed so that phenylglyoxal was specific for arginine residues. These properties of the compound led to its use for the investigation of a possible catalytic role for arginine residues in DNA polymerase I.

The evidence obtained from Lineweaver–Burk and Hill plots in conjunction with binding and affinity-label studies showed the presence of single reactive lysine and arginine residues in or about the polymerizing active center of DNA polymerase I.

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[1] Abbreviations used are: MMPR-OP, β-D-ribosyl-6-methylthiopurine oxidation product; PG, phenylglyoxal; NCMIA, *N*-carboxymethylisatoic anhydride.