JOURNAL OF VIROLOGY, Mar. 1977, p. 831-842

Vol. 21, No. 3
Printed in U.S.A.

Amount and Distribution of Virus-Specific Sequences in Giant RNA Molecules Isolated from Polyoma-Infected Mouse Kidney Cells

ZE'EV LEV AND HAIM MANOR*

Department of Biology and The Joseph and Arlene Taub Institute of Biological Research, Technion-Israel Institute of Technology, Haifa, Israel

Received for publication 16 August 1976

A two-step hybridization with polyoma DNA was used to study the composition of giant RNA molecules synthesized in mouse kidney cells late in productive infection by polyoma virus. Giant molecules longer than a complete transcript of the polyoma genome were purified from cells that had been pulse-labeled for 30 min with [^{3}H]uridine and annealed, under mild conditions (50% formamide, 37°C), with polyoma DNA loaded on nitrocellulose filters. Hybridized RNA {6 to 7% of the entire population of ^{3}H-labeled molecules and up to 15% of the molecules containing polyadenylic acid [poly(A)]} was eluted and annealed a second time with polyoma DNA under more stringent conditions. In this second step, 75% of the ^{3}H-labeled RNA formed an RNase-resistant hybrid. Under the same conditions, complementary RNA hybridized with polyoma DNA to a maximal extent of 80%. Since the difference between 75 and 80% is within the experimental error of the hybridization assay, it is inferred that the giant molecules selected by the first hybridization may consist entirely of virus-specific sequences or contain, at the most, a minor fraction of nonviral sequences. To examine the possibility that such nonviral sequences are clustered at the 3'-terminus of these molecules, poly(A)$^+$ giant RNA, which had not been preselected by hybridization with polyoma DNA, was fragmented by a limited alkaline hydrolysis. Fragments linked to the poly(A) segment were separated from the rest of the cleavage products. A one-step hybridization with polyoma DNA revealed that both fractions contain 8 to 10% of virus-specific sequences. These results indicate that the 3'-termini of the poly(A)$^+$ polyoma-specific giant RNA molecules consist of viral rather than nonviral sequences.

Mouse kidney cells infected with polyoma virus synthesize, late in infection, giant RNA molecules (>30S) containing virus-specific sequences. These molecules are longer than a complete transcript of the virus genome (1, 3, 24). There are several ways by which virus-specific giant RNA may be produced. (i) RNA polymerase may read the circular virus DNA more than once. (ii) The giant chains may be transcription products of an oligomer consisting of several virus DNA monomers joined in tandem. (iii) These giant RNA molecules may be transcribed from a template consisting of virus DNA integrated into the host genome, as well as adjacent chromosomal sites. Transcription may begin at a virus DNA site and extend into adjacent chromosomal DNA, or vice versa. The three possibilities are not mutually exclusive. For example, giant RNA may be transcribed from a circular DNA consisting of viral as well as host sequences (11, 12).

Transcription from integrated virus and host DNA has been widely discussed as a likely possibility, in view of several reports on the integration of polyoma DNA, and DNA of the closely related simian virus 40, into chromosomal DNA during productive infection and in transformed cells (2, 14, 23). Furthermore, it has been reported that giant RNA synthesized in simian virus 40-infected and transformed cells consists of covalently linked host and viral sequences (9, 25, 29).

In agreement with Acheson et al. (1), we have found that giant RNA molecules synthesized in polyoma-infected mouse kidney cells contain up to 15% virus-specific sequences. In view of the above considerations, it was interesting to determine whether these sequences are all found in giant chains that are mainly or entirely virus specific, or instead are distributed among chains that also contain a major fraction of nonviral sequences. In this article, we describe the results of two types of experiments bearing on this question.

In one type of analysis, giant RNA molecules containing virus-specific sequences were isolated by hybridization with polyoma DNA. The hybridization reaction was carried out under mild conditions (50% formamide, 37°C) to minimize shearing of the chains. Subsequently, the proportion of viral sequences in these selected chains was determined by a second hybridization with polyoma DNA, carried out under more stringent conditions. The entire population of giant RNA molecules and a subclass of molecules containing polyadenylic acid [poly(A)] were separately analyzed by this two-step hybridization technique. A second type of experiment was designed to examine more closely the 3'-termini of the molecules containing poly(A). These molecules were cleaved into 10 times smaller fragments by a limited alkaline hydrolysis. Fragments linked to the poly(A) segment, and therefore representing the 3'-termini of the chains, were separated from the rest of the fragments. The concentration of virus-specific sequences was determined in each of these two fractions by a one-step hybridization with polyoma DNA. The results of both types of analysis are discussed in relation to the models described above.

MATERIALS AND METHODS

Cells and growth media. Mouse kidney cell cultures were prepared from 8-day-old C3H mice. The kidneys were removed, washed with phosphate-buffered saline (5), and cut into small pieces with scissors. The tissue derived from 150 kidneys was suspended in a 50-ml pancreatin solution, prepared by dissolving one tablet of buffered pancreatin (Oxoid, code BR1) in 50 ml of water. The suspension was incubated for 10 min at 37°C; during the incubation period the tissue was further disrupted by pipetting. Next, the cells were removed by decantation, added to a centrifuge tube containing 10 ml of calf serum, and stored at 4°C. The remaining tissue was treated with pancreatin, and the cells were decanted and added to the serum, as described above. The same procedure was repeated twice. Next, the tube was centrifuged for 2 min at 2,000 rpm, and the cells were suspended in Dulbecco modified Eagle medium supplemented with 10% calf serum and 0.025 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer. Cells derived from one kidney were seeded in a 9-cm Falcon plate in 10 ml of medium. The plates were incubated at 37°C in an atmosphere containing 95% air + 5% CO_2. We thank Sven Warnaar for communicating this procedure to us.

Growth and purification of polyoma virus. Polyoma virus was grown in mouse kidney cells. Stocks of virus were obtained by injecting 10^6 PFU into 3- to 5-day-old mice. Cultures of kidney cells were prepared from these mice after 1 week, as described above. Ten days later, the cell lysates were collected, and the virus was purified according to Winocour (33). The virus used for injection had been propagated in this way for several years. Plaque-purified virus stocks were prepared as described by Manor and Neer (15). The experiments reported in this article were conducted with a plaque-purified virus, except where specified otherwise.

Virus infection and radioactive labeling of RNA. Mouse kidney cell cultures were infected with polyoma virus at a multiplicity of infection of 50, 3 days after reaching confluence at 6×10^6 cells/9-cm Falcon plate. The medium was removed, and virus suspended in 0.5 ml of phosphate-buffered saline supplemented with 1% calf serum was added to each plate. The plates were incubated for 1.5 h at 37°C and then supplemented with 9 ml of Dulbecco medium containing 0.025 M HEPES and 5% calf serum. Thirty hours postinfection, the medium was replaced with 3 ml of the above medium containing dialyzed calf serum, to which was added 0.28 mCi of [5-^{3}H]uridine per ml (specific activity, 23 Ci/mmol). After 30 min, the medium was removed and the cells were washed with phosphate-buffered saline at 4°C. For labeling with ^{32}P, the cells were preincubated for 2 h with a mixture of a complete medium and a phosphate-free medium at a ratio of 1:9. At the end of the preincubation period, this medium was replaced with a phosphate-free medium containing 0.25 mCi of ^{32}P per ml. The cells were incubated and harvested as described above.

Extraction of RNA. RNA was extracted from virus-infected or uninfected cells by the method of Parish and Kirby (20). The cells in each plate were lysed at 4°C with 5 ml of an aqueous solution containing 0.2 M NaCl, 1% (wt/vol) sodium triisopropylnaphthalene sulfonate, 6% (wt/vol) sodium *p*-aminosalicylate, and 6% (vol/vol) 2-butanol. The suspension was mixed with an equal volume of a water-saturated solution of phenol and cresol (55:8) containing 3 mg of 8-hydroxyquinoline. After shaking for 10 min, the phases were separated by centrifugation; the aqueous phase was brought to 0.50 M NaCl and extracted again with the same solution. After two additional extractions, twice the volume of ethanol was added, and the solution was left overnight at −20°C. The RNA was sedimented in a Sorvall centrifuge for 1 h at 10,000 rpm and 4°C. The precipitated RNA was washed with 10 ml of a solution containing ethanol and 0.01 M Tris (pH 7.4) at a ratio of 2:1 (vol/vol) and then dried with a stream of N_2. Recovery of pulse-labeled RNA exceeded 80%.

Glycerol gradient centrifugation of RNA. The RNA was precipitated in ethanol and dried under a stream of N_2. The precipitate was dissolved in a solution containing 0.01 M Tris (pH 7.4) and 0.001 M EDTA and heated for 10 min at 65°C. Two milliliters of an RNA solution was layered on top of a glycerol gradient (20 to 50%, 36 ml), which also contained 0.10 M NaCl, 0.01 M EDTA, and 0.05 M Tris, pH 7.4. The gradient was centrifuged in the SW27 rotor of a Spinco ultracentrifuge, as specified in the legends to the figures. Fractions were collected, and samples were assayed for trichloroacetic acid-insoluble radioactivity, as described by Manor and Neer (15). The total yield of giant RNA (>30S) was about 10^6 cpm/plate. The giant RNA recovered from the gra-

dient was precipitated in ethanol and dried. The precipitate was dissolved in 0.40 ml of 98% distilled dimethyl sulfoxide (Me_2SO) also containing 0.01 M Tris (pH 7.4) and 0.001 M EDTA, and the solution was incubated for 5 min at 25°C. Next, the RNA and Me_2SO were diluted five times by adding 1.6 ml of 0.01 M Tris (pH 7.4) and centrifuged in a glycerol gradient, as described above.

Chromatography in oligo(dT)-cellulose columns. The RNA was precipitated in ethanol, dried with a stream of N_2, and dissolved in 0.10 ml of a solution containing 0.01 M Tris, pH 7.4, 0.01 M EDTA, 0.10% sodium dodecyl sulfate (SDS), and 0.50 M NaCl (ETS + 0.50 M NaCl). The solution was mixed with 20 mg (dry weight) of oligodeoxythymidylate [oligo(dT)]-cellulose preequilibrated with the same buffer. The mixture was incubated for 5 min at 23°C and then was poured into a small column and washed first with 10 ml of the above buffer at 23°C (fraction I). Next, the column was washed at 23°C with 4 ml of ETS buffer containing 0.05 instead of 0.50 M NaCl (ETS + 0.05 M NaCl) and then with 2 ml of 0.05 M NaCl, 0.01 M Tris, pH 7.4 (fraction II). The final washing was carried out at 45°C with 5 ml of 0.01 M Tris, pH 7.4 (fraction III).

Nitrocellulose filter binding. Nitrocellulose filter binding was carried out as described by Gorski et al. (7). Fraction III from the oligo(dT)-cellulose column was brought to 0.50 M KCl and 0.001 M $MgCl_2$ (KTM buffer). The solution was filtered slowly through a membrane filter (type HAWP, 0.45-nm pore size; Millipore Corp.) and then washed with 5 ml of the same buffer at 23°C. The bound RNA was eluted by soaking the filter in 1 ml of 0.01 M Tris (pH 7.4) at 23°C and shaking for 30 min. The soaking and shaking was repeated 3 times.

Selection of RNA containing polyoma-specific sequences. Nitrocellulose filters (Millipore type HAWP, 0.45-nm pore size; diameter, 13 mm) were loaded with polyoma DNA, as described by Gillespie and Spiegelman (6). The filters were incubated for 3 h at 37°C in a solution containing 0.05 M Tris (pH 7.4), 0.75 M NaCl, 0.01 M EDTA, 0.10% SDS, 0.05% Ficoll, 0.05% polyvinylpyrrolidone, and 0.05% bovine serum albumin before being used for hybridization (4). Blank filters were similarly treated. Giant RNA was precipitated in ethanol, dried with a stream of N_2, and dissolved in 0.20 ml of a solution containing 0.01 M Tris (pH 7.4), 0.15 M NaCl, 0.002 M EDTA, 0.02% SDS, and 90% formamide. The solution was incubated for 5 min at 23°C and then diluted with an appropriate buffer, to a volume of 0.40 ml, of the following final composition: 0.05 M Tris, pH 7.4; 0.75 M NaCl; 0.01 M EDTA; 0.10% SDS; 500 μg of yeast RNA per ml; and 50% formamide (32). This solution was incubated for 16 h at 37°C in a vial containing one filter loaded with 1 μg of polyoma DNA and a blank filter. At the end of the incubation period the filters were removed from the vial and washed on each side with a solution containing 3× SSC (SSC is 0.15 M NaCl, 0.015 M trisodium citrate) and 0.10% SDS at 65°C. Next, the filters were placed in 1.0 ml of hybridization buffer that does not contain RNA and then incubated for 1 h at 37°C and washed again as described above. The RNA was eluted by vigorously shaking the filters for 15 min at 50°C in an 0.50-ml solution containing 0.01 M Tris (pH 7.4), 0.001 M EDTA, and 90% formamide. The elution step was repeated twice. The eluants were combined, diluted fourfold with 0.01 M Tris (pH 7.4), and centrifuged for 5 min at 10,000 rpm and 4°C in a Sorvall centrifuge to remove contaminating particles.

RNA-DNA hybridization in aqueous solution. The procedure developed by Gillespie and Spiegelman (6) was used for hybridization. Each filter (Millipore type HAWP, 0.45-nm pore size; diameter, 13 mm) contained 1.5 μg of polyoma DNA prepared from a plaque-purified virus stock, as described by Manor and Neer (15). Giant RNA was cleaved into smaller fragments by a limited alkaline hydrolysis prior to hybridization, such that the assays were always conducted with fragments of equal size. The RNA was incubated in 0.50 ml of 4× SSC at 65°C for 20 h in a vial containing a filter loaded with virus DNA and a blank filter containing no DNA. At the end of the incubation period, each filter was washed on each side with 50 ml of 3× SSC at 65°C. The filters were then incubated for 1 h at 37°C in 1 ml of 2× SSC containing 40 μg of RNase A and 20 U of RNase T_1. Next, the filters were washed with 100 ml of 3× SSC at 65°C, dried, and counted as described by Manor and Neer (15).

RNA cleavage. RNA was incubated for 5 min at 4°C in a solution containing 0.20 N NaOH. At the end of the incubation period, the solution was neutralized with an equivalent concentration of HCl and an equal volume of 1 M Tris, pH 7.4, and then the RNA was precipitated in ethanol, as described above.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was carried out essentially as described by Nakazato et al. (19). Poly(A) or RNA was precipitated in ethanol. The pellet was dissolved in 0.10 ml of a buffer containing 0.04 M Tris-acetate (pH 7.5), 0.02 M sodium acetate, and 0.002 M EDTA. The solution was incubated for 5 min at 65°C, cooled to room temperature, and subjected to electrophoresis in a 6-cm-long 10% polyacrylamide gel at 80 V and 4.5 mA/gel. The gel was cut into 2-mm slices, each of which was taken into a 1-ml solution of 30% H_2O_2 and boiled for 15 min. Next, 5 ml of a solution containing a toluene scintillation mix (14) and Triton, at a ratio of 2:1, was added, and the mixture was counted in a Packard Tri-Carb spectrometer.

Chemicals. [^{3}H]uridine was purchased from the Israel Nuclear Center. Oligo(dT)-cellulose was from Collaborative Research Inc. Polyuridylic acid [poly(U)] was obtained from Miles Laboratories. RNase A was from Worthington; RNase T_1 and poly(A) were from Sigma. Sodium triisopropylnaphthalene-sulfonate was purchased from Eastman Kodak Co., and sodium-*p*-aminosalicylate was from K & K Labs. Me_2SO from Fluka was distilled before use. Formamide was obtained from Merck. Pancreatin (code BR_1) was from Oxoid, yeast RNA labeled with [^{3}H]adenosine was kindly supplied by H. Shalitin, Department of Biology, Technion, and synthetic ^{32}P-labeled oligo(A) was a gift of G. Kaufmann,

Weizmann Institute of Science [for details on synthesis of the oligo(A), see reference 10].

RESULTS

Isolation of giant RNA molecules. Mouse kidney cells were infected with polyoma virus. Thirty hours postinfection, the cells were labeled with [^{3}H]uridine for 30 min. An equal number of uninfected mouse kidney cells was labeled for 30 min with $^{32}PO_4$. The uninfected and infected cells were lysed, the lysates were combined, and RNA was extracted from the mixture. The method developed by Parish and Kirby (20) was used for cell lysis and RNA extraction, because it yields, in our hands, a larger product than the SDS-hot phenol method (27). To dissociate possible aggregates, the RNA was heat treated, as described in Materials and Methods. This treatment has been found to dissociate mRNA from giant nuclear RNA, without causing a significant degradation of the latter (16). Giant RNA was isolated by centrifugation in a glycerol gradient. Figure 1A shows a typical sedimentation profile, in which 74% of the acid-insoluble ^{32}P and ^3H radioactivity, representing pulse-labeled RNA from the uninfected and infected cells, respectively, appears in molecules whose sedimentation coefficient is equal to or larger than 30S. This population of RNA molecules includes the 45S and 32S ribosomal precursor species and large heterogeneous nuclear RNA (27). Note that in these gradients more radioactivity appears in the 32S than in the 45S rRNA. We have found that in RNA extracted from cells that have been labeled for 10 or 20 min with [^{3}H]uridine, the 45S species is the predominant peak (not shown).

The fractions denoted in Fig. 1A by a horizontal bar were pooled, and the RNA was precipitated in ethanol and dried. The precipitated RNA was dissolved in 98% Me_2SO to disrupt residual aggregates (28) and recentrifuged in a glycerol gradient. Figure 1B shows the sedimentation profile obtained after the second centrifugation. It can be seen that although the average sedimentation coefficient was somewhat reduced, most of the RNA still moved faster than the 28S rRNA species. The fractions denoted by the horizontal bar were pooled and used for a further analysis.

Alkaline cleavage of giant RNA. In some of the experiments reported below, the giant molecules were cleaved into smaller fragments by a limited alkaline hydrolysis, as described in Materials and Methods. Figure 2 shows the sedimentation profile of one preparation of giant RNA (>40S, preselected) before and after

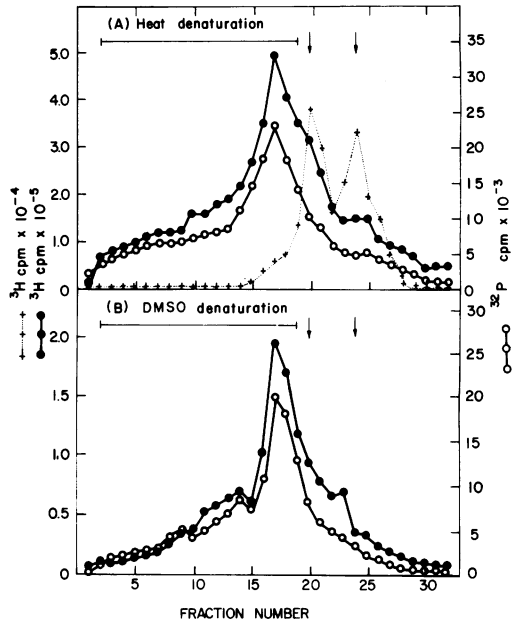

FIG. 1. *Glycerol gradient centrifugation of RNA from virus-infected and uninfected cells. (A) RNA from 6 × 10^6 polyoma-infected cells labeled for 30 min with [^{3}H]uridine late in infection, and from 6 × 10^6 uninfected cells labeled for 30 min with ^{32}P, was heat treated and centrifuged in a glycerol gradient, as described in Materials and Methods. Centrifugation was performed at 22,000 rpm and 4°C for 15 h. Giant RNA was pooled from the fractions denoted by the horizontal bar. 18S and 28S rRNA markers were centrifuged in parallel gradients. Sedimentation was from right to left. Symbols: (●) ^{3}H-labeled RNA; (○) ^{32}P-labeled RNA; (+) ^{3}H-labeled rRNA markers. (B) Giant RNA isolated from the gradient shown in (A) was ethanol precipitated, treated with Me_2SO, and centrifuged, as described in Materials and Methods. The fractions denoted by the horizontal bar were pooled and further analyzed. The arrows denote the positions attained by the rRNA markers.*

cleavage in alkali, as determined by centrifugation in a sucrose gradient. The average sedimentation coefficient of the cleaved product is estimated as 14S.

Isolation of poly(A)$^+$ RNA. Up to 40% of the giant RNA molecules synthesized in mammalian cells contain a segment of poly(A) whose length varies between 150 and 200 nucleotides (31). The poly(A) is covalently linked to the 3'-termini of these molecules. Some giant molecules also contain a nonterminal oligo(A) sequence of about 30 nucleotides (19). In the present experiments, RNA chains containing poly(A) [poly(A)$^+$ RNA] were separated from chains containing oligo(A) and from those that contain neither poly(A) nor oligo(A) [poly(A)$^-$ RNA] by oligo(dT)-cellulose column chromatog-

raphy. The RNA was adsorbed to oligo(dT)-cellulose, which was subsequently packed into a column. Elution was carried out in three steps, yielding three fractions (fractions I, II, and III), as described in Materials and Methods. Poly(A)$^-$ RNA was eluted in step I, and RNA containing short oligo(A) segments was eluted in step II. Poly(A)$^+$ RNA was eluted in step III. In some experiments, the poly(A)$^+$ RNA was further purified by adsorption to a nitrocellulose filter, according to Gorski et al. (7). These authors have demonstrated that RNA dissolved in a high-ionic-strength buffer is bound to a nitrocellulose filter, if, and only if, it contains a poly(A) segment whose length exceeds 50 nucleotides. Therefore, this procedure should provide additional fractionation of RNA molecules containing poly(A) from those containing short oligo(A) segments.

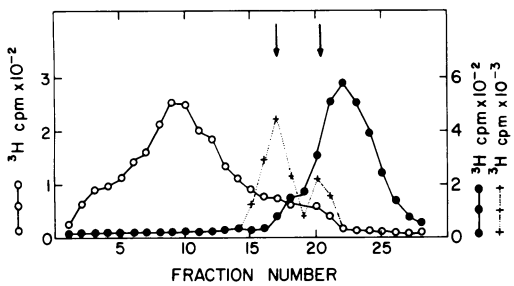

FIG. 2. *Sucrose gradient centrifugation of giant RNA before and after alkaline cleavage. Giant RNA was cleaved by treatment with alkali, as described in Materials and Methods. Aliquots of the original sample* (○) *and the cleaved product* (●) *were each centrifuged in a sucrose gradient (15 to 30%) containing 0.10 M NaCl, 0.01 M EDTA, and 0.01 M Tris, pH 7.4. Centrifugation in an SW50 rotor was for 3 h at 45,000 rpm and 4°C. 18S and 28S rRNA markers* (+) *were centrifuged in parallel gradients. Sedimentation was from right to left.*

Tables 1 and 2 present experiments that confirm the specificity of these fractionation procedures. In the first two experiments presented in Table 1, total RNA from cells labeled for 30 min with [^{3}H]uridine, or giant RNA isolated by glycerol gradient centrifugation, was chromatographed on oligo(dT)-cellulose columns (step 1). About 8% of the radioactively labeled chains were eluted in both cases as fraction III, and hence could be classified as poly(A)$^+$ RNA. In many similar experiments, we have found that the poly(A)$^+$ RNA molecules never exceeded 15% of the giant RNA (see Discussion). Next, samples of the poly(A)$^+$ chains isolated from each column were either rechromatographed on an oligo(dT)-cellulose column or adsorbed to a nitrocellulose filter (step 2). It could be seen that in both experiments over 75% of the poly(A)$^+$ RNA chains were eluted as fraction

TABLE 1. *Selection of poly(A)$^+$ RNA*

Expt	Type of RNA[a]	Step 1: chromatography on an oligo(dT)-cellulose column[b]			Step 2: rechromatography on an oligo(dT)-cellulose column or nitrocellulose filter binding					
		Input (cpm)	% Eluted as fraction: II	% Eluted as fraction: III	Fraction analyzed[c]	Input for oligo(dT)-cellulose column chromatography (cpm)	% Eluted as fraction: II	% Eluted as fraction: III	Input for nitrocellulose filter binding assay (cpm)[d]	% Bound to the filter
1	Total	1.91 × 10^6	2.5	8.4	III	9,000	11.5	85.2	30,000	70.2
					III + poly(A)	9,000	6.6	1.5	30,000	4.7
2	Giant	1.34 × 10^6	1.9	8.1	III	27,000	9.2	77.0	1,600	90.0
3	Poly(A)$^+$ giant cleaved with alkali	88,400	2.0	6.3	III	1,000	9.8	86.6	1,000	75.0
					III + poly(A)		ND[e]	ND	1,000	7.7
					III + poly(U)		ND	ND	1,000	9.7

[a] The "total" RNA consists of at least 70% giant RNA (Fig. 1). "Giant" refers to RNA purified by two successive glycerol gradient centrifugations, as shown in Fig. 1. Poly(A)$^+$ giant RNA was isolated by glycerol gradient centrifugation followed by oligo(dT)-cellulose column chromatography and then was cleaved with alkali. The cleaved product was used in experiment 3 for step 1.

[b] Carried out as described in Materials and Methods.

[c] Samples from the RNA eluted in the first chromatography as fraction III were rechromatographed or assayed by nitrocellulose filter binding. In the competition experiments with poly(A), 200 μg of poly(A) was adsorbed to a column or filter, as described in Materials and Methods; subsequently, the RNA samples were applied to the same column (or filter). In the competition experiment with poly(U), the RNA was preincubated for 30 min at 37°C in 0.30 ml of KTM buffer plus 150 μg of poly(U) per ml before being adsorbed to the filter.

[d] Carried out as described in Materials and Methods. Less than 2% of the poly(A)$^-$ giant RNA binds to the filters under the conditions used for these assays.

[e] ND, Not determined.

TABLE 2. *Oligo(dT)-cellulose column chromatography and nitrocellulose filter binding of mouse RNA, yeast RNA, and synthetic oligo (A)*

Type of RNA[a]	Chromatography on oligo(dT)-cellulose column				Nitrocellulose filter binding	
	Input (cpm)	% Eluted as fraction:			Input (cpm)	% Bound to filter
		II	III	Ratio III/II		
Mouse	7,800	15.4	72.0	4.7	7,000	97.2
Yeast	4,000	28.1	41.9	1.5	4,000	25.5
Oligo(A)	1,800	87.0	3.6	0.04		ND[b]

[a] ^{3}H-labeled total mouse RNA, or yeast RNA, was chromatographed on an oligo(dT)-cellulose column; fractions II and III were combined in each case, and the mixture was used for both assays. The ^{32}P-labeled oligo(A) (15 to 30 nucleotides) was a gift of G. Kaufmann (10).

[b] ND, Not determined.

III, and 70 to 90% were bound to the filter. In the third experiment, giant poly(A)$^+$ RNA was first isolated and fragmented by a limited alkaline hydrolysis; then the cleavage products were chromatographed on an oligo(dT)-cellulose column (step 1). It could be seen that 6.3% of the fragments were eluted as fraction III. These results would be expected if each poly(A)$^+$ giant molecule were cleaved into at least 10 fragments, only one of which contained poly(A). In step 2, over 80% of the poly(A)$^+$ fragments were eluted from the column as fraction III, and 75% were bound to the filter.

Table 1 also shows that preloading the columns, or the filters, with a synthetic poly(A) effectively blocks subsequent binding of the RNA and that preincubating the RNA with poly(U) inhibits binding to the filters. Other experiments (not shown) revealed that preincubation with poly(U) also inhibits binding of the poly(A)$^+$ RNA to oligo(dT)-cellulose.

Table 2 presents oligo(dT)-cellulose column chromatography and nitrocellulose filter binding assays of mouse RNA, yeast RNA, and synthetic oligo(A) [the mouse and yeast RNA had been chromatographed on oligo(dT)-cellulose columns; fractions II and III from these columns were combined and used for the assay). It can be seen that whereas over 70% of the mouse RNA molecules were eluted as fraction III, 87% of the oligo(A) chains (which consist of 15 to 30 nucleotides) were eluted as fraction II. The proportion of the yeast RNA eluted as fraction II is also significantly higher than the corresponding proportion of the mouse RNA. It is to be noted that yeast RNA molecules contain poly(A) segments whose average length is only 50 nucleotides. The molecules eluted as fraction II presumably contain oligo(A) segments smaller than this average value (17). The distribution of the mouse RNA among the two fractions is in line with the size of its poly(A), estimated to be about 200 nucleotides (see Fig. 3). The data on the binding of the mouse RNA and the yeast RNA to the nitrocellulose filters are in line with the above conclusions.

Figure 3 illustrates the results of an experiment designed to examine the separation of poly(A) from oligo(A) and to characterize the poly(A) segment in mouse nuclear RNA. In this experiment, mouse nuclear RNA labeled with ^{32}P was hydrolyzed by treatment with pancreatic and T_1 RNase. The RNase-resistant material was chromatographed on an oligo(dT)-cellulose column. About 50% of the radioactivity bound to the column was eluted as fraction II. The rest of the radioactivity, eluted as fraction III, was passed through a nitrocellulose filter, as described above. Three different fractions eluted from the column and the filter were characterized by polyacrylamide gel electrophoresis. Figure 3A presents an electrophoretogram of the material eluted from the column as fraction II. This material consists of polynucleotide chains whose length is estimated to be smaller than 80 nucleotides. Figure 3B shows that the length of the poly(A) chains eluted from the column as fraction III and then bound to the filter is about 200 nucleotides. Base analysis performed by alkaline hydrolysis, followed by ion-exchange column chromatography (8), revealed that these chains consist of at least 88% AMP. Figure 3C shows that the material eluted from the column as fraction III, but not adsorbed to the filter, consists of heterogeneous species whose length is smaller than 80 nucleotides. A comparison of Fig. 3B and C reveals that only 10% of the material eluted as fraction III were not bound to the filter. These results confirm the observation already made in the experiments presented in Table 2 that the oligo(dT)-cellulose chromatography (with the three-step elution) itself provides a rather efficient separation of poly(A) from oligo(A).

Distribution of virus-specific sequences

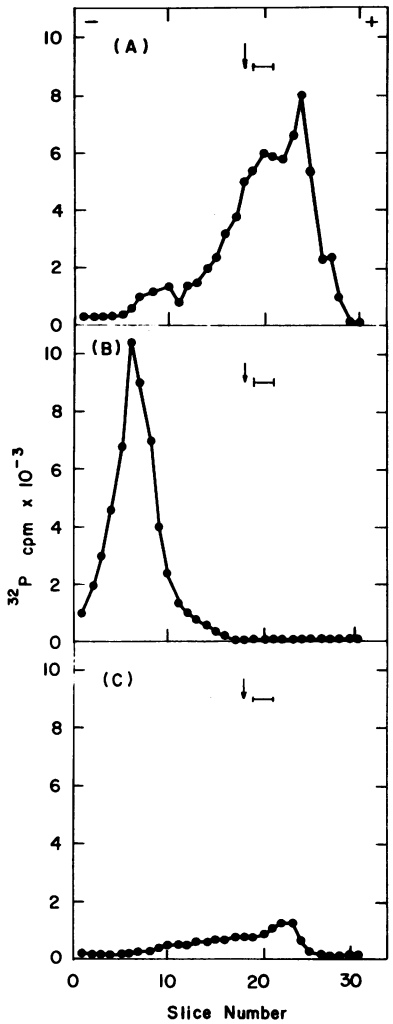

FIG. 3. *Polyacrylamide gel electrophoresis of poly(A) and oligo(A) isolated from mouse nuclear RNA. Mouse kidney cells infected with polyoma virus were labeled for 2 h with ^{32}P at 300 μCi/ml, as described in Materials and Methods. Nuclei were isolated by the method of Lindberg and Darnell (13). Nuclear RNA was extracted and precipitated in ethanol. The RNA was dissolved in 0.01 M Tris, pH 7.4, containing 0.30 M NaCl and digested for 1 h at 37°C with 40 μg of RNase A and 20 U of RNase T_1 per ml. Next, $MgCl_2$ and DNase were added to a final concentration of 2 mM and 10 μg/ml, respectively, and the solution was incubated for 15 min at 37°C. The digest was extracted once with a mixture of phenol, chloroform, and isoamyl alcohol (50:49:1, by volume). The RNase-resistant residue was precipitated in ethanol, dissolved in ETS + 0.50 M NaCl, and chromatographed on an oligo(dT)-cellulose column. The material recovered as fraction II was subjected to electrophoresis, as described in Materials and Methods (A). Poly(A) recovered as fraction III was further fractionated by adsorption to a nitrocellulose filter. The fraction bound to the filter (B) and the fraction that did not bind to the filter (C) were subjected to electrophoresis as described above. The horizontal bars denote the position in the gels attained by bromophenol blue. The arrows denote the position attained by 4S RNA subjected to electrophoresis in parallel. Background of 10 cpm was subtracted from each fraction.*

among giant RNA molecules. A two-step hybridization with polyoma DNA was used to isolate and characterize the poly(A)$^+$ giant RNA molecules containing virus-specific sequences. Figure 4 is a diagrammatic representation of this procedure. A mixture of ^{3}H-labeled giant RNA from polyoma-infected cells and ^{32}P-labeled giant RNA from an equal number of uninfected cells was purified by two successive centrifugations in glycerol gradients, as described above. The poly(A)$^+$ molecules were isolated by oligo(dT)-cellulose column chromatography and hybridized with polyoma DNA fixed on a nitrocellulose filter. The hybridization reaction was performed under mild conditions (50% formamide, 37°C) to minimize shearing of the chains. RNA specifically bound to the DNA-containing filter was eluted and cleaved into smaller fragments by a limited alkaline hydrolysis. The resulting fragments were hybridized again with polyoma DNA under more stringent conditions (aqueous salt solution, 65°C, RNase treatment) to determine the proportion of virus-specific sequences in the selected molecules.

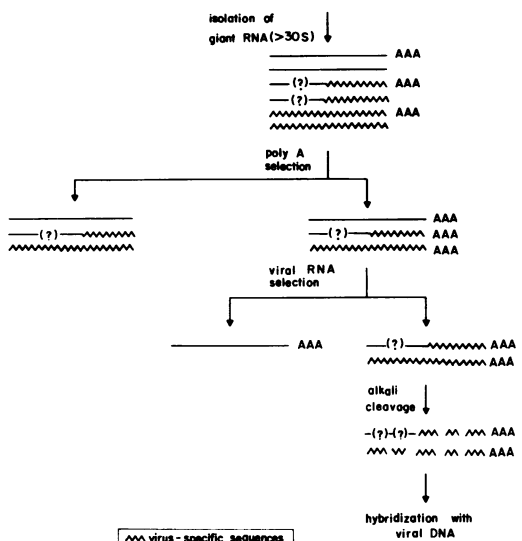

FIG. 4. *Diagrammatic representation of the procedure for purification and analysis of virus-specific giant RNA.*

The results of one experiment of the type illustrated in Fig. 4 are summarized in Table 3 (experiment 1). It can be seen that during the first hybridization, 15.4% of the ^{3}H-labeled poly(A)$^+$ giant RNA molecules were bound to the filter containing polyoma DNA, and 0.10% were bound to the blank filter. Of the ^{32}P-labeled RNA, 0.26% hybridized with polyoma DNA and 0.05% were bound to the blank filter. The binding of the ^{32}P-labeled RNA from uninfected cells to the filter containing polyoma DNA could result from an interaction between these molecules and the RNA from the virus-specific cells, which specifically hybridized with polyoma DNA. However the ^{3}H/^{32}P ratio indicates that this nonspecific binding accounts for less than 1/73 (<2%) of the ^{3}H-labeled RNA bound to the polyoma DNA filter. It is also noteworthy that under the conditions used for hybridization, the presence of poly(A) does not by itself lead to an appreciable binding of the giant molecules to the filter. We infer from these results that at least 98% of the ^{3}H-labeled RNA molecules bound to the polyoma DNA filter contain virus-specific sequences.

Although the first hybridization was performed under mild conditions, some degradation of giant molecules could have occurred during the course of the reaction. In this case, nonviral "tails," possibly found in molecules that hybridized with polyoma DNA (Fig. 4), would have been split. Such an artefact would lead in a second hybridization to an overestimation of the proportion of virus-specific sequences in the selected molecules. To examine this possibility, the molecules that did not react with polyoma DNA during the first hybridization, and therefore remained in the hybridization mixture, and the virus-specific molecules that did hybridize, and were subsequently eluted from the filter, were centrifuged in glycerol gradients. Figure 5A shows that most of the unhybridized RNA sediments faster than the 28S rRNA species. This result indicates that little, if any, RNA degradation has taken place during the course of the hybridization reaction. However, the virus-specific RNA was found to sediment at a slower rate (Fig. 5B). Hence, the virus-specific molecules may have been degraded to some extent during elution from the filter. Another possibility is that the molecules containing virus-specific sequences are, in fact, somewhat smaller than the rest of the giant molecules. Nevertheless, over one-third of the polyoma-specific RNA sediments faster than one complete transcript of the virus genome, whose sedimentation coefficient is estimated as 26S.

For the second hybridization step, giant virus-specific molecules were pooled from the fractions in the gradient shown in Fig. 5B denoted by the horizontal bar. The ratio of DNA/RNA in this reaction was chosen such that the amount of hybridized RNA was maximal, as demonstrated by control experiments in which the ratio was tripled and no further increase in hybridization was observed (not shown). It can be seen that 75% of the preselected giant RNA specifically hybridized with polyoma DNA. As discussed above, the conditions used for the

TABLE 3. *Two-step hybridization analysis of virus-specific giant RNA*[a]

Expt	Type of RNA[b]	Step 1: selection					Step 2: analysis of selected RNA[c]	
		Input (cpm)	% of input hybridized[d]	Ratio ^{3}H/^{32}P in hybridized RNA[e]	% of input eluted	Ratio ^{3}H/^{32}P in eluted RNA	Input (cpm)	% of input hybridized
1	Poly(A)$^+$ giant RNA							
	Infected, ^{3}H	7.15×10^5	15.4 (0.10)	73	7.9 (0.01)	60	726	75.0 (<0.03)
	Uninfected, ^{32}P	1.33×10^6	0.26 (0.05)		0.14 (0.01)			
2	Total giant RNA							
	Infected, ^{3}H	1.18×10^6	6.4 (0.09)	20.5	5.4 (0.06)	18.4	1,873	75.6 (0.02)
	Uninfected, ^{32}P	3.22×10^5	0.40 (0.09)		0.36 (0.07)			
3	^{3}H-labeled cRNA						4,240	79.7 (0.02)

[a] A diagrammatic representation of this experiment is given in Fig. 4, and a description is given in the text. Experimental details are presented in Materials and Methods.

[b] In both experiments, a plaque-purified virus stock was used for infection. Pulse-labeling with [^{3}H]uridine was for 30 min. In experiments 1 and 2, labeling with ^{32}P was for 2 h and 30 min, respectively. Polyoma ^{3}H-labeled cRNA was prepared as described in reference 14.

[c] The input consists of giant molecules selected from the eluted RNA after another glycerol gradient centrifugation (see Fig. 5).

[d] The numbers in parentheses represent, in all cases, the percentage of input bound to a blank filter.

[e] The ratios were calculated after subtracting the blank values.

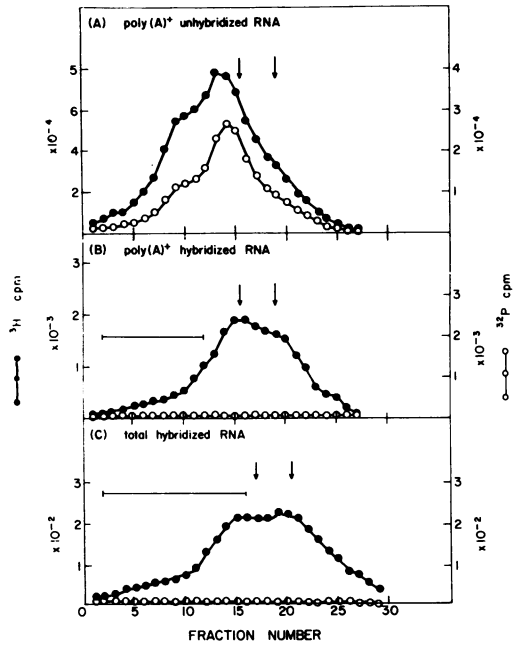

FIG. 5. *Glycerol gradient centrifugation of RNA selected by hybridization with polyoma DNA. Poly(A)$^+$ giant RNA was annealed, under mild conditions (50% formamide, 37°C), with polyoma DNA loaded on a nitrocellulose filter. RNA that did not hybridize (A), and RNA that did hybridize and was later eluted from the filter (B), were centrifuged in a glycerol gradient, as described in Materials and Methods. Total [poly(A)$^+$ and poly(A)$^-$] giant RNA, hybridized with and later eluted from a polyoma DNA filter, was also centrifuged in a glycerol gradient (C). Centrifugation was at 25,000 rpm and 4°C for 16 h. Sedimentation was from right to left. The arrows denote the positions attained by 18S and 28S rRNA markers centrifuged in parallel gradients.*

second hybridization assay were rather stringent. Thus, even the extent of hybridization of complementary RNA (cRNA), which consists of virus-specific sequences exclusively, does not exceed 80% (Table 3, experiment 3). Taking into consideration the experimental error of the assay, we conclude, therefore, that the selected giant molecules may consist entirely of virus-specific sequences or contain, at the most, a minor fraction of nonviral sequences.

In the above experiment, only the poly(A)$^+$ giant RNA molecules were analyzed by the two-step hybridization technique. It was interesting to carry out a similar analysis of the entire population of giant RNA molecules, most of which do not contain poly(A). The results of one experiment of this type are shown in Table 3 (experiment 2) and Fig. 5C. Giant RNA molecules, which had not been fractionated by oligo(dT)-cellulose column chromatography, were annealed with polyoma DNA. Only 6.4% of these molecules hybridized with the virus DNA, compared to 15.4% in the previous experiment. This lower value is probably due to the presence of the ribosomal precursor species, which do not contain poly(A), in the unfractionated RNA preparation. It is also noteworthy that during the selection step nonspecific binding of ^{32}P-labeled RNA from uninfected cells to the polyoma DNA filter was higher in this experiment than in the previous experiment. This result could stem from the fact that the present RNA preparation was not chromatographed on oligo(dT)-cellulose, a procedure that removes contaminating DNA. Nevertheless, the nonspecific binding accounts for less than 6% of the binding of the ^{3}H-labeled RNA. Figure 5C shows the sedimentation profile of the RNA eluted from the polyoma DNA filter after the first hybridization. It is similar to the profile of the polyoma-specific poly(A)$^+$ RNA shown in Fig. 5B. The fractions denoted by the horizontal bar were pooled and annealed a second time with polyoma DNA. It can be seen that over 75% of the RNA hybridized with the DNA. Thus, the poly(A)$^-$ virus-specific giant RNA molecules, like the poly(A)$^+$ molecules previously analyzed, contain, at the most, a minor fraction of nonviral sequences.

Analysis of the composition of the 3′-termini in poly(A)$^+$ giant RNA molecules. The above results do not exclude the presence of a small fraction of nonviral sequences in the polyoma-specific giant RNA molecules. In particular, such nonviral sequences could occupy a specific portion in these molecules. One way to examine this question would be to isolate specific parts of the chains and to find out whether the ratio of viral to nonviral sequences in these parts differs from the average ratio. We have carried out such an analysis of the 3′-terminal portion of the poly(A)$^+$ giant RNA molecules.

Figure 6 presents an outline of the experiment designed for this purpose. ^{3}H-labeled giant RNA (>40S) is purified by two successive glycerol gradient centrifugations, as described above. Poly(A)$^+$ chains are isolated by oligo(dT)-cellulose column chromatography, followed by adsorption to a nitrocellulose filter (see above). The purified molecules are cleaved by a limited alkaline hydrolysis into about 10 times smaller fragments. Fragments containing poly(A) are separated from the rest of the fragments, as described above. Each of these two fractions is hybridized with polyoma DNA under exhaustive conditions to determine the proportion of virus-specific sequences. It is to be noted that in this experiment the giant mole-

cules containing virus-specific sequences were not preselected by hybridization in formamide. Preselection is not necessary in this case because the polyoma-specific sequences account for about 1/10 of all sequences in the pulse-labeled giant RNA, whereas the background of the reaction, determined by binding of the RNA to blank filters is less than 1/5,000 of the input. Indeed, it is estimated that hybridization carried out in one step is sufficiently sensitive to detect a difference of 10% in the concentration of virus-specific sequences between two RNA preparations.

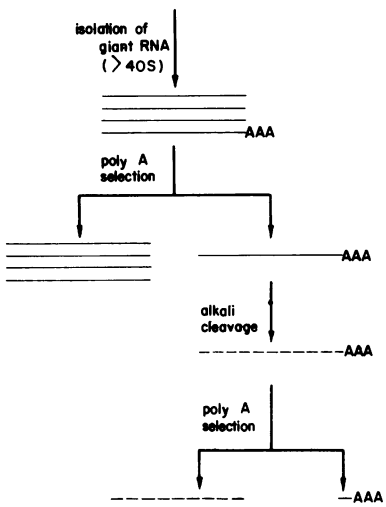

FIG. 6. *Diagrammatic representation of the procedure for isolation of 3'-terminal fragments of giant RNA.*

The experiment described above can distinguish between two possibilities. If giant molecules, which consist of virus-specific sequences over most of their length, contain nonviral sequences at the 3'-end, then the poly(A)$^+$ fragments are expected to hybridize with polyoma DNA less efficiently than the rest of the fragments. If, on the other hand, in most or all of these chains the 3'-termini contain virus-specific sequences, then the poly(A)$^+$ fragments are expected to hybridize with polyoma DNA as efficiently as the rest of the fragments.

Table 4 presents the results obtained in two independent experiments of this type. In the first experiment, the cells were infected with a virus that had been propagated for several years, as described by Winocour (33). The polyoma stock used for the second experiment was prepared from a virus that had been twice plaque purified (15). The results of both experiments can be summarized as follows. (i) The poly(A)$^+$ and poly(A)$^-$ giant RNA contain 8 to 10% polyoma-specific sequences. This result differs from the results presented in Table 3, in which the poly(A)$^+$ giant RNA was found to consist of a higher proportion of virus-specific molecules than the unfractionated RNA [which contains predominantly poly(A)$^-$ molecules]. This difference may be due to the presence of both the 32*S* and the 45*S* rRNA species in the unfractionated RNA preparation, used in the previous experiment, whereas the present poly(A)$^-$ preparation contains only the 45*S* species. (ii) The poly(A)$^+$ and poly(A)$^-$ fragments, derived from the same poly(A)$^+$ giant RNA molecules, contain about the same proportion of polyoma-specific sequences as the parent mole-

TABLE 4. *Analysis of 3'-terminal fragments of giant RNA by a one-step hybridization with polyoma DNA*

Type of infecting virus[a]	Type of RNA[b]	Poly(A)$^-$ RNA			Poly(A)$^+$ RNA		
		Input (cpm of ^{3}H)	RNA hybridized		Input (cpm of ^{3}H)	RNA hybridized	
			cpm of ^{3}H[c]	% of input		cpm of ^{3}H	% of input
Virus propagated by injection	Giant	3,890	330(1)	8.5	2,570	214(0)	7.8
	Fragments	2,680	213(0)	7.9	1,580	155(0)	9.2
	Small	2,980	100(5)	3.2	2,760	142(1)	5.1
Plaque-purified virus	Giant	960	86(0)	9.0	580	63(0)	10.8
	Fragments	1,470	162(0)	10.5	300	26(0)	8.7
	Small	2,600	122(1)	4.7	2,380	211(0)	8.7

[a] Details on the preparation of the virus stocks are presented in Materials and Methods.

[b] The poly(A)$^-$ and poly(A)$^+$ fragments were both derived from the poly(A)$^+$ giant chains (see Fig. 6). Small RNA refers to RNA molecules whose sedimentation coefficient is in the range of 8 to 16*S*, isolated from a gradient such as that shown in Fig. 1A.

[c] The numbers in parentheses refer to the radioactivity bound to blank filters. Filters loaded with *E. coli* DNA bound as much RNA as the blank filters. Background radioactivity of 7 cpm was subtracted from all values.

cules. (iii) Poly(A)$^-$ "small" RNA (8-16S RNA molecules) isolated from the first glycerol gradient used to prepare giant RNA, contains about half the proportion of polyoma-specific sequences as the giant RNA. Poly(A)$^+$ small RNA isolated from the same gradient is enriched with polyoma-specific sequences. These results indicate that findings (i) and (ii) are due neither to a failure of our fractionation procedures to select for molecules containing virus-specific sequences nor to an artefact of the hybridization assays.

DISCUSSION

Our results show that about 70% of the pulse-labeled RNA recovered from mouse kidney cells late in productive infection by polyoma virus sediment as giant molecular species (>30S). These giant molecules, which include the 32S and 45S rRNA precursors, contain about 8% virus-specific sequences. The intracellular concentration of giant RNA molecules may be higher than the observed yield, because molecules of this size are extremely labile and may have been degraded during extraction and subsequent manipulation. Indeed, Parish and Kirby's extraction procedure used in the present experiments yields, in our hands, larger quantities of giant RNA than the SDS-hot phenol method. Another improvement in technique may provide a higher yield of giant molecules. The intracellular location of the virus-specific giant molecules has not been determined in the present experiments. However, previous work has indicated that only nuclei contain polyoma-specific RNA of a comparable size (3). The biological function of these molecules is still obscure. One possibility would be that the giant chains are precursors to the smaller cytoplasmic polyoma mRNA (3); a similar function was postulated for heterogeneous nuclear RNA in uninfected cells (18).

It has been reported that up to 40% of the giant RNA synthesized in various mammalian cells contain poly(A) (31). In our RNA preparations, only 8 to 15% of the giant molecules contain poly(A). This discrepancy can be accounted for by the presence of larger amounts of the 32S and the 45S rRNA species [which do not contain poly(A)] in our preparations, because in previous experiments rRNA synthesis was inhibited by incubating the cells in the presence of low concentrations of actinomycin D (21). Furthermore, in our experiments the RNA is labeled for a short period (30 min) and therefore includes molecules whose synthesis or processing has not been completed. These molecules may not contain poly(A), because the latter is synthesized, several minutes after transcription is completed, by a stepwise addition of adenylate residues to the 3'-end (22). Our results indicate that 10 to 15% of the poly(A)$^+$ and about 8% of the poly(A)$^-$ giant RNA molecules contain virus-specific sequences, in agreement with the results of Rosenthal et al. (24). The corresponding fraction among the nonribosomal poly(A)$^-$ molecules cannot be estimated because the ratio of rRNA/non-rRNA has not been determined.

The experiments reported in Results demonstrate that about 75% of the sequences in the giant RNA molecules selected by the first hybridization step form RNase-resistant hybrids with polyoma DNA in the second hybridization. Under the same conditions, polyoma cRNA, which consists entirely of virus-specific sequences, hybridizes with polyoma DNA to a maximal extent of 80%. Since the difference of 5% is within the experimental error of the hybridization assay, these results are compatible with the possibility that the selected giant molecules are entirely composed of virus-specific sequences. Such molecules could be transcribed from extrachromosomal monomeric or oligomeric virus DNA templates (see Introduction).

We have recently carried out an analysis of virus-specific sequences in giant RNA molecules isolated from polyoma-infected cells, using as probes the separated strands of restriction endonuclease fragments of polyoma DNA. Our preliminary results indicate that these molecules contain approximately equimolar proportions of sequences transcribed from the entire L strand of the polyoma genome. These results are consistent with the above conclusions. A similar analysis of a mixture of 16S and 19S RNA species, isolated from the same gradient used for preparing giant RNA, reveals that, as expected, these species contain transcripts of the "late" half of the polyoma genome and no "anti-late" RNA. Therefore, the polyoma-specific giant RNA cannot consist of aggregates of these smaller RNA molecules (Lev, Manor, and Kamen, unpublished data).

However, the presence of a minor fraction (<20%) of nonviral (e.g., host) sequences in the virus-specific giant RNA molecules has not been entirely excluded by any of the above results. It is to be noted, though, that most of these chains are over 1.5 times longer than a complete transcript of the polyoma genome and hence cannot be transcribed from a template consisting of one polyoma DNA molecule integrated into chromosomal DNA. Instead, RNA chains of this size, which contain less than 20% host sequences, would have to be transcribed from a template consisting of at least two virus

DNA molecules inserted within the host DNA.

The experiments reported in Results show that the concentration of polyoma-specific sequences in the 3'-termini of the poly(A)$^+$ giant RNA chains is equal to the average concentration of viral sequences along these chains. This result excludes the possibility that the 3'-termini of the polyoma-specific giant RNA molecules, which contain poly(A), consist of nonviral sequences. Molloy et al. (18) proposed that mammalian mRNA is derived from the 3'-termini of heterogeneous nuclear RNA molecules. In view of this hypothesis, it would be interesting to find out whether the 3'-terminal portion of the virus-specific giant RNA molecules consists of sequences found in polyoma mRNA species.

ACKNOWLEDGMENTS

This work was supported by a grant from the Leukemia Research Foundation and grants from the Ben Gurion Foundation of the Histadrut and The Israel Commission for Basic Research.

We wish to thank Rachel Heinish and Yaffa Bot for able technical assistance.

LITERATURE CITED

1. **Acheson, N. H., E. Buetti, K. Scherrer, and R. Weil.** 1971. Transcription of the polyoma virus genome: synthesis and cleavage of giant late polyoma-specific RNA. Proc. Natl. Acad. Sci. U.S.A. 68:2231-2235.
2. **Aloni, Y., E. Winocour, L. Sachs, and J. Torten.** 1969. Hybridization between SV40 DNA and cellular DNA's. J. Mol. Biol. 44:333-345.
3. **Buetti, E.** 1974. Characterization of late polyoma mRNA. J. Virol. 14:249-260.
4. **Denhardt, D. T.** 1966. A membrane filter technique for the detection of complementary DNA. Biochem. Biophys. Res. Commun. 23:641-646.
5. **Dulbecco, R., and M. Vogt.** 1954. Plaque formation and isolation of pure lines with poliomyelitis viruses. J. Exp. Med. 99:167-182.
6. **Gillespie, D., and S. Spiegelman.** 1965. A quantitative assay for DNA-RNA hybrids with DNA immobilized on a membrane. J. Mol. Biol. 12:829-842.
7. **Gorski, J., M. R. Morrison, C. G. Merkel, and J. B. Lingrel.** 1974. Size heterogeneity of polyadenylate sequences in mouse globin messenger RNA. J. Mol. Biol. 86:363-371.
8. **Hayashi, M., and S. Spiegelman.** 1961. The selective synthesis of informational RNA in bacteria. Proc. Natl. Acad. Sci. U.S.A. 47:1564-1580.
9. **Jaenisch, R.** 1972. Evidence of SV40 specific RNA containing virus and host specific sequences. Nature (London) New Biol. 235:46-72.
10. **Kaufmann, G., M. Fridkin, A. Zutra, and U. Z. Littauer.** 1971. Monofunctional substrates of polynucleotide phosphorylase. Eur. J. Biochem. 24:4-11.
11. **Lavi, S., and E. Winocour.** 1972. Acquisition of sequences homologous to host deoxyribonucleic acid by closed circular simian virus 40 deoxyribonucleic acid. J. Virol. 9:309-316.
12. **Lavi, S., and E. Winocour.** 1974. Accumulation of closed-circular polyoma DNA molecules containing host DNA during serial passage of the virus. Virology 57:296-299.
13. **Lindberg, U., and J. E. Darnell.** 1970. SV40-specific RNA in the nucleus and polyribosomes of transformed cells. Proc. Natl. Acad. Sci. U.S.A. 65:1089-1096.
14. **Manor, H., M. Fogel, and L. Sachs.** 1973. Integration of viral into chromosomal deoxyribonucleic acid in an inducible line of polyoma-transformed cells. Virology 53:175-185.
15. **Manor, H., and A. Neer.** 1975. Effects of cycloheximide on virus DNA replication in an inducible line of polyoma-transformed rat cells. Cell 5:311-318.
16. **McKnight, G. S., and R. T. Schimke.** 1974. Ovalbumin messenger RNA: evidence that the initial product of transcription is the same size as polysomal ovalbumin messenger. Proc. Natl. Acad. Sci. U.S.A. 71:4327-4331.
17. **McLaughlin, C. S., J. R. Warner, M. Edmonds, H. Nakazato, and M. H. Vaughan.** 1973. Polyadenylic acid sequences in yeast messenger ribonucleic acid. J. Biol. Chem. 248:1466-1471.
18. **Molloy, G. R., N. Jelinek, M. Salditt, and J. E. Darnell.** 1974. Arrangement of specific oligonucleotides within poly A terminated HnRNA molecules. Cell 1:43-52.
19. **Nakazato, H., M. Edmonds, and D. W. Kopp.** 1974. Differential metabolism of large and small poly A sequences in the heterogeneous nuclear RNA of Hela cells. Proc. Natl. Acad. Sci. U.S.A. 71:200-204.
20. **Parish, J. H., and K. S. Kirby.** 1966. Reagents which reduce interactions between ribosomal RNA and rapidly-labeled RNA from rat liver. Biochim. Biophys. Acta 129:554-562.
21. **Perry, R. P., J. R. Greenberg, and K. D. Tartof.** 1970. Transcription of ribosomal heterogeneous nuclear and messenger RNA in eucaroyotes. Cold Spring Harbor Symp. Quant. Biol. 35:577-587.
22. **Perry, R. P., D. E. Kelly, and J. LaTorre.** 1974. Synthesis and turnover of nuclear and cytoplasmic polyadenylic acid in mouse L cells. J. Mol. Biol. 82:315-331.
23. **Ralph, R. K., and J. S. Colter.** 1972. Evidence for the integration of polyoma virus DNA in a lytic system. Virology 48:49-58.
24. **Rosenthal, L. J., C. Salomon, and R. Weil.** 1976. Isolation and characterization of poly A-containing intranuclear polyoma-specific "giant" RNA's. Nucleic Acids Res. 5:1167-1183.
25. **Rozenblatt, S., and E. Winocour.** 1972. Covalently linked cell and SV40-specific sequences in an RNA from productively infected cells. Virology 50:558-566.
26. **Sambrook, J., J. H. Westphal, P. R. Srinivasan, and R. Dulbecco.** 1968. The integrated state of viral DNA in SV40-transformed cells. Proc. Natl. Acad. Sci. U.S.A. 60:1288-1295.
27. **Scherrer, K., and J. E. Darnell.** 1962. Sedimentation characteristics of rapidly labeled RNA from HeLa cells. Biochem. Biophys. Res. Commun. 7:486-490.
28. **Strauss, J. H., R. N. Kelly, and R. L. Sinsheimer.** 1968. Denaturation of RNA with dimethylsulfoxide. Biopolymers 6:793-807.
29. **Wall, R., and J. E. Darnell.** 1971. Presence of cell and virus specific sequences in the same molecules of nuclear RNA from virus-transformed cells. Nature (London) New Biol. 232:73-76.
30. **Weil, R., C. Salomon, E. May, and P. May.** 1974. A simplifying concept in tumour virology: virus-specific pleiotropic effectors. Cold Spring Harbor Symp. Quant. Biol. 39:381-395.
31. **Weinberg, R. A.** 1973. Nuclear RNA metabolism. Annu. Rev. Biochem. 42:329-354.
32. **Weinberg, R. A., S. O. Warnaar, and E. Winocour.** 1972. Isolation and characterization of simian virus 40 ribonucleic acid. J. Virol. 10:193-201.
33. **Winocour, E.** 1963. Purification of polyoma virus. Virology 19:158-168.