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Isolation and Characterization of Simian Virus 40 Ribonucleic Acid

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Deoxyribonucleic acid-ribonucleic acid (RNA) hybridization in formamide was used to isolate simian virus 40-specific RNA. Early in the lytic cycle, a 19S viral RNA species was observed. Late in the lytic cycle, 16S and 19S viral species were found. The 16S and 19S species of viral RNA were localized in the cytoplasm. High-molecular-weight heterogeneous RNA, containing viral sequences, was isolated from the nuclear fraction of infected cells late in the lytic cycle. This RNA may contain nonviral sequences linked to viral sequences. The formamide hybridization technique can be used to isolate intact late lytic viral RNA which is at least 99% pure.

During simian virus 40 (SV40) lytic infection of monkey kidney cells, host-cell ribonucleic acid (RNA) metabolism continues at a normal or even an increased rate (16). Early in the lytic cycle, viral RNA constitutes only 0.01 to 0.1% of the total RNA made in the cell. Later in infection, 2 to 3% of the total RNA synthesized is hybridizable to viral deoxyribonucleic acid (DNA; 2, 17, 19). Analysis of viral RNA metabolism is thus complicated by continued host synthesis. Since various attempts to suppress selectively host RNA metabolism have proven unsuccessful, DNA-RNA hybridization is at present the only technique for the resolution and analysis of SV40 RNA metabolism.

We have utilized the technique of DNA-RNA hybridization in formamide (13) to produce undegraded and relatively pure SV40 homologous RNA for analysis of viral RNA metabolism. Labeled RNA from infected cells is hybridized in 50% formamide (13) to DNA immobilized on cellulose nitrate filters. The filters are washed free from nonhybridized material; the hybridized RNA is eluted from the filters with a high concentration of formamide and is subsequently analyzed on sucrose gradients or polyacrylamide gels. The RNA is undegraded after such treatment, and RNA of a molecular weight up to several million can be produced.

In BS-C-1 monkey cells infected at a multiplicity of 50 to 100 plaque-forming units per cell, viral DNA synthesis begins about 18 hours postinfection, and cytopathic effects appear about 24 hr later (2). The RNA produced during the lytic cycle has been divided into "early" RNA, which is made before the onset of viral DNA synthesis, and "late" RNA, made after DNA synthesis has begun. The amount of viral RNA made late in the infectious cycle is about 40 times greater than the amount of early RNA (2). The RNA made late in the infection contains sequences homologous to 75 to 100% of the viral genome; the early RNA contains only about one-third as many sequences (2, 14, 17, 19, 20).

This report presents some of the properties of the different classes of viral RNA isolated by the formamide hybridization technique. First, the properties of the homogeneously sedimenting viral RNA species present in large amount in the cytoplasm late in the infection are described. Second, we examined early RNA, both by labeling infected cells early in the lytic cycle and by labeling cells later in the cycle in the presence of inhibitors of DNA synthesis. Finally, we investigated properties of the high-molecular-weight heterogeneous nuclear RNA produced by the virus late in the lytic cycle.

MATERIALS AND METHODS

RNA preparation. Cells were washed three times in phosphate-buffered saline and then lysed with lysis buffer [1% (w/v) sodium dodecyl sulfate (SDS), 0.001 M ethylenediaminetetraacetate (EDTA), 0.1 M NaCl, 0.1% polyvinyl sulfate, 0.01 M tris(hydroxymethyl)aminomethane (Tris), *p*H 7.4], which is identical to SDS buffer (18) but with the addition of polyvinyl sulfate and the doubling of the SDS concentration. The lysate was extracted at 25 C by use of the phenol-chloroform-isoamyl alcohol procedure (18). This "cold" phenol procedure extracted 95% of

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the virus-specific RNA. Only a further 5% was extractable from the cold phenol interface by subsequent hot phenol (65 C) extraction. After two cycles of phenol-chloroform-isoamyl alcohol extraction, the nucleic acids in the aqueous phase were precipitated at −20 C with 2 volumes of ethanol. The precipitate, collected by centrifugation (15 min, 15,000 rev/min in a Sorvall RC2 centrifuge), was resuspended in 2 ml of SDS buffer. A 400-μg amount of nonradioactive monkey liver RNA was added as carrier, and the RNA was precipitated by adding LiCl to 2 M (in later experiments, the carrier RNA was omitted). The RNA precipitate was collected as above, and the LiCl precipitation was repeated. By this procedure, 99% of the DNA was removed. The RNA was finally precipitated by 2 volumes of ethanol. When the RNA was to be analyzed by acrylamide gel electrophoresis, the extract was treated with Worthington electrophoretically purified deoxyribonuclease (10 μg/ml, 37 C, 5 min, in 0.05 M NaCl, 0.01 M $MgCl_2$, and 0.01 M Tris, *p*H 7.4). Acrylamide gel electrophoresis was performed as previously described (26); the 2.6% acrylamide gels of 15 cm length were run for 9 hr at 75 v. Cell fractionation was performed by the hypotonic swelling-Dounce homogenization technique (18; the nuclei were washed with 1% Nonidet P40 or Tween 40). Sucrose gradient sedimentation of isolated RNA was performed in 15 to 30% (w/w) sucrose gradients made with SDS buffer (18). Dimethyl sulfoxide (DMSO) centrifugation (22) was performed in a gradient of 5 to 20% (w/v) sucrose in a solvent of 99% DMSO.

Formamide hybridization. Precipitated RNA was centrifuged and resuspended in 0.6 ml of hybridization buffer [0.75 M NaCl, 0.5% (w/v) SDS, 50% (v/v) formamide (Fluka), 0.01 M Tris, *p*H 7.9]. The salt and formamide concentrations were taken from the work of McConaughy et al. (13). A similar hybridization procedure has been reported previously by others (6). The solution of RNA was incubated at 37 C for 18 hr with 5 to 10 μg of DNA immobilized on a membrane filter (7). The SV40 DNA used for hybridization was prepared as before (2) from CsCl band purified virus (3). After incubation, the filter was withdrawn from the solution, washed under suction with 50 ml of SSC (0.15 M NaCl, 0.015 M sodium citrate) containing 0.5% (w/v) SDS. The filter was then incubated with 10 ml of hybridization buffer for 2 hr at 37 C. Finally, each side of the filter was washed under suction with 20 ml of SSC and 10 ml of hybridization buffer. To recover hybridized RNA from the filter, the filter was incubated at 37 C for 1 hr in 1 ml of elution buffer (90 volumes of formamide, 9 volumes of distilled water, 1 volume of SDS buffer). The eluate was collected, and the eluted RNA was precipitated by the addition of 100 μg of yeast transfer RNA, NaCl to 0.2 M, and 2 volumes of ethanol.

RESULTS

Late lytic homogeneous RNA species. The sedimentation profile of virus-specific RNA from BS-C-1 cells labeled 30 to 40 hr postinfection is shown in Fig. 1. Total cellular RNA was isolated

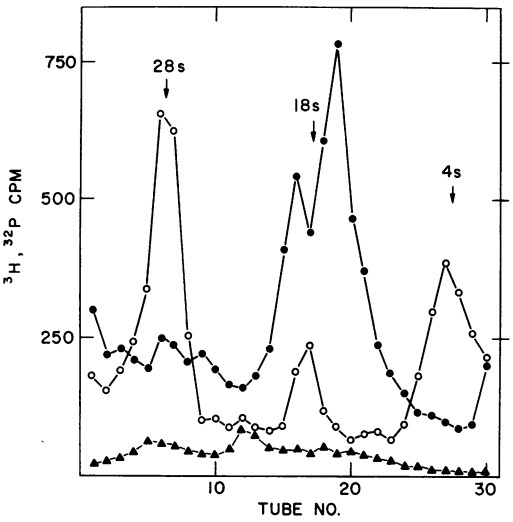

FIG. 1. *Sedimentation profile of the virus-specific RNA isolated from BS-C-1 cells late in the lytic cycle. BS-C-1 cells were infected with SV40 (100 plaque-forming units per cell) or mock-infected with medium alone, and labeled with ^3H-uridine (25 μCi/ml; 28 Ci/mmole) between the 30th and 40th hr postinfection. The labeled RNA species were isolated from the infected cells, hybridized in formamide to SV40 DNA, and eluted as described in Materials and Methods. The eluted RNA was layered over a gradient of SDS-sucrose (15 to 30%) and centrifuged for 16 hr at 25,000 rev/min in a Spinco SW25.1 rotor at 25 C. Fractions were counted directly in Bray's scintillation fluid. Symbols: ●, ^3H-RNA from infected cells; ▲, ^3H-RNA from mock-infected cells; ○, ^{32}P-ribosomal RNA marker (contains also 4S RNA).*

and then hybridized in formamide to SV40 DNA immobilized on a cellulose nitrate filter. The hybridized RNA was eluted from the filter and analyzed by sucrose gradient band sedimentation. Two apparently homogeneous species of RNA, which sediment at 16*S* and 19*S*, were consistently found. The small amount of material sedimenting at less than 16*S* suggests that only a minimal amount of degradation occurred during the hybridization and elution steps. Depending upon labeling and extraction procedures, some heterogeneous material sedimenting at greater than 20*S* was also observed. No such species of RNA were detected in mock-infected cultures (Fig. 1).

When the eluate from the formamide hybridization step was analyzed by electrophoresis through polyacrylamide gels (Fig. 2a), the two main species of viral RNA were resolved to a greater degree. However, the RNA species which sedimented at 19*S* relative to the ribosomal markers migrated in the gel like RNA with an apparent sedimentation coefficient of about 22*S*. Polyoma

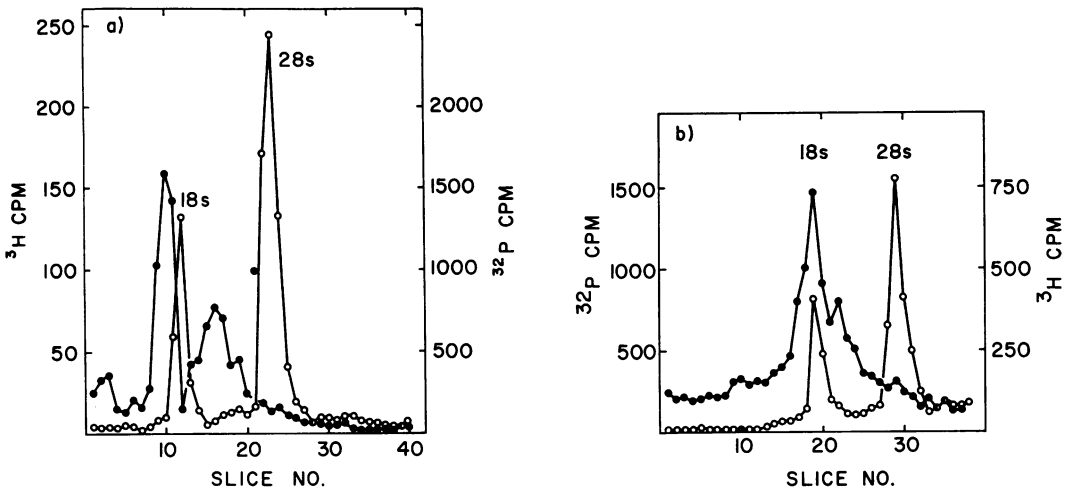

FIG. 2. *Acrylamide gel analysis of virus-specific RNA. ^{3}H-RNA was isolated from cells infected and labeled as in Fig. 1. (a) A sample was hybridized to SV40 DNA in formamide, eluted, and applied to an acrylamide gel for electrophoresis (Materials and Methods). (b) Another sample of the ^{3}H-RNA was fractionated on an acrylamide gel without prior hybridization to SV40 DNA. After gel electrophoresis, each gel fraction was incubated in 2 × SSC for 18 hr at 65 C with a filter containing 1 μg of SV40 DNA. The filters were treated for 1 hr with 20 μg of pancreatic ribonuclease, dried, and counted in toluene scintillation fluid. Symbols: ●, ^{3}H-viral RNA; ○, ^{32}P-ribosomal RNA marker.*

virus RNA showed an electrophoretic profile almost identical to that shown in Fig. 2a (R. Weinberg, *unpublished data*). To determine the possible effects of the formamide hybridization procedure on the size and relative amounts of the two species, we applied whole cellular RNA, isolated from cells late in the infectious cycle, directly to an acrylamide gel without prior hybridization. The RNA from each fraction of the gel was hybridized independently to filter-bound SV40 DNA (Fig. 2b). Although the data in Fig. 2b clearly confirm the existence of two RNA species, they have slightly different electrophoretic mobilities, perhaps owing to the exposure of the first sample (Fig. 2a) to the denaturing effects of formamide before gel electrophoresis.

A subsequent report examines in detail two properties of the 16*S* and 19*S* RNA species which affect their relative proportion and homogeneity as seen in the figures here. First, the 19*S* late lytic species is metabolically much more labile than the 16*S*. Second, the size of each of these species decreases with time, a phenomenon contributing to their band width in some of the gradient and gel profiles shown here. This latter phenomenon may derive from a decrease in the size of the polyadenylic acid tail of these RNA species as the messenger RNA molecules become older (R. A. Weinberg, *in preparation*). These two phenomena will not be examined further in this report.

The sedimentation rates in SDS-sucrose gradients of the two species shown in Fig. 1 are stable after recentrifugation, reextraction with phenol, and rehybridization of the RNA. The RNA in Fig. 3a was prepared as in Fig. 1. Peak fractions from each of the two species were pooled and resedimented (Fig. 3b and 3c). Each RNA species resedimented at the same rate as originally observed. The peak tubes from these gradients (Fig. 3b and 3c) were pooled, precipitated, resuspended in lysis buffer, and then reextracted with phenol-chloroform-isoamyl alcohol in the presence of unlabeled, infected BS-C-1 cells. After reextraction, each RNA was rehybridized in formamide to SV40 DNA, eluted, and resedimented. Each RNA species continued to sediment at the rate initially observed (Fig. 3d and 3e). Thus, the two RNA species are not two conformations of the same RNA species in equilibrium with one another. Their sedimentation rate is unaffected by the second cycle of sedimentation, extraction, and hybridization. Other experiments (not shown) indicated that these viral RNA species retain their sedimentation rates if they are denatured with 90% DMSO (9) before being sedimented through a sucrose density gradient. Also, the two RNA species co-sedimented approximately with 18*S* ribosomal RNA when centrifuged in 99% DMSO (22). They cannot be separated from each other, however, because of the low resolution afforded by DMSO gradients, but both appear to have molecular weights of at least 650,000.

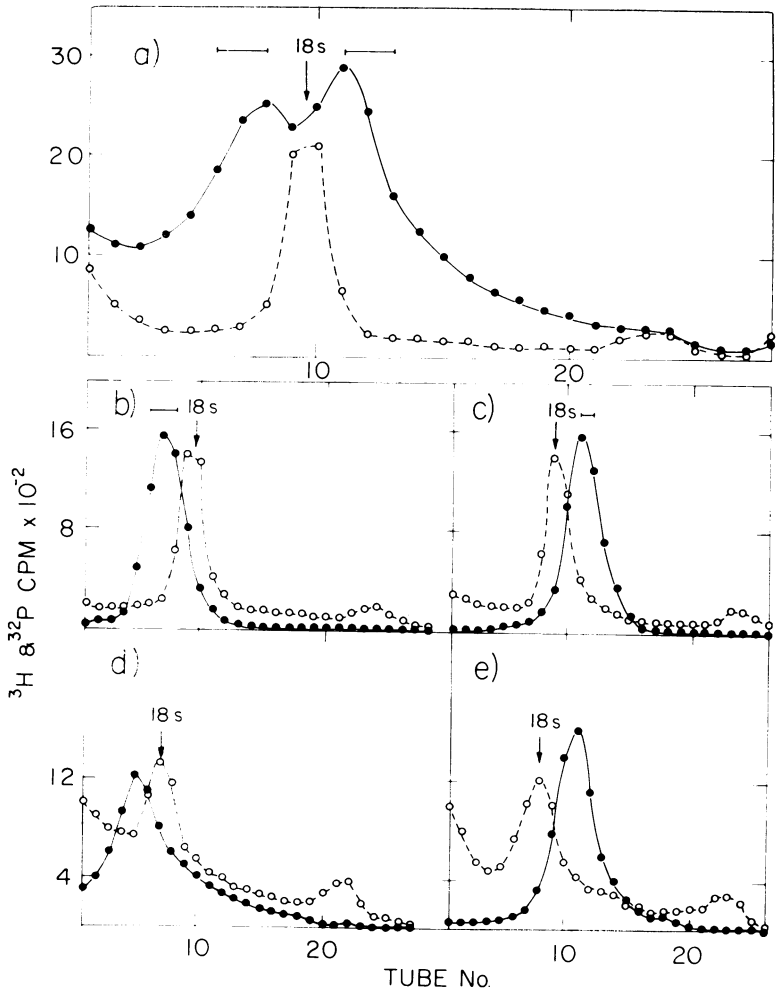

FIG. 3. *Sedimentation of 16S and 19S viral RNA after additional processing. Ten cultures of BS-C-1 cells were infected with SV40 and labeled with ^3H-uridine (500 μCi/ml, 15 C/mmole) from 33 to 44 hr postinfection. (a) The infected cells were lysed in RSB (18) containing 1% Nonidet P40, and the cytoplasmic virus-specific RNA species were extracted, purified by hybridization to SV40 DNA in formamide, and characterized by centrifugation through 15 to 30% SDS-sucrose gradients (Materials and Methods). The sedimentation conditions used here were 34,000 rev/min, 15 hr, in a Spinco SW41 rotor. (b) Fractions 6, 7, and 8 of panel a were pooled and the RNA was re-centrifuged as above. (c) Fractions 11, 12, and 13 of panel a were pooled, and centrifuged as above. (d and e) Fractions 6, 7, and 8 of panel b were pooled as the 19S species; fractions 11 and 12 of panel c were pooled as the 16S species. The RNA from these two pools was precipitated with ethanol, and redissolved in 3 ml of lysis buffer. To each of two cultures of unlabeled SV40-infected BS-C-1 cells, at 44 hr postinfection, was added 1.5 ml of the 19S RNA in lysis buffer; the 16S RNA in lysis buffer was similarly added to two other unlabeled, SV40-infected cultures. The RNA was extracted with phenol-chloroform-isoamyl alcohol, hybridized to SV40 DNA, in formamide, recovered from the hybrid complex, and centrifuged in 15 to 30% SDS-sucrose as above. (d) 19S RNA. (e) 16S RNA. Symbols: ●, ^3H-viral RNA; ○, ^{32}P-ribosomal RNA marker.*

The formamide hybridization-elution technique yields viral RNA virtually free from host contamination. Cytoplasmic RNA from infected cells produces 120 times more radioactivity eluted from the viral DNA filters after hybridization than does cytoplasmic RNA from mock-infected cells. This eluted RNA thus has less than 1% host RNA contamination. Another measure of the specificity of the formamide procedure is provided by Table 1. RNA from the nuclear and cytoplasmic fractions of SV40-infected cells isolated late in infection was first hybridized in formamide to SV40 DNA filters. The hybridized RNA was then eluted, divided into portions, and

TABLE 1. *Specificity of the formamide hybridization procedure*[a]

Origin of ^{3}H-RNA	Formamide hybridization to SV40 DNA (10 μg)		Rehybridization of eluate in 6 × SSC at 65 C			
	^{3}H-RNA incubated (counts/min)	^{3}H-RNA bound to and eluted from filter (counts/min)	^{3}H-RNA incubated (counts/min)	DNA on filter	^{3}H-RNA bound to filter (counts/min)	Input hybridized (%)
SV40-infected cells						
Nuclear fraction.....	9.2×10^6	5.6×10^4	1.3×10^4	SV40 (10 μg)	3,000	23.0
			1.3×10^4	BS-C-1 (10 μg)	400	3.1
			1.3×10^4	Blank	76	0.6
Cytoplasmic fraction.....	13.1×10^6	1.6×10^5	4.5×10^4	SV40 (10 μg)	19,000	41.5
			4.5×10^4	BS-C-1 (10 μg)	470	1.0
			4.5×10^4	Blank	120	0.3

[a] The infection, labeling, and cell fractionation are described in the legend to Fig. 6. The eluates of the first cycle of formamide hybridization were rehybridized by the Gillespie and Spiegelman (7) procedure: 20 hr, 65 C in 6 × SSC, followed by ribonuclease treatment of 20 μg/ml, 1 hr at room temperature.

rehybridized by the standard Gillespie-Spiegelman technique (7) to SV40 DNA, BS-C-1 DNA, or blank filters containing no DNA. The eluted cytoplasmic RNA hybridized with high efficiency back to SV40 DNA. The nuclear RNA, significantly, hybridized less efficiently back to SV40 DNA (see below).

Early lytic RNA synthesis. Early RNA is defined as the class of viral RNA species which is synthesized from the beginning of the infectious cycle to the onset of DNA synthesis. Previous workers have shown that, in the presence of inhibitors of DNA synthesis, late RNA synthesis and late functions are suppressed (4, 10, 20), whereas early RNA continues to be synthesized (20). Thus, late RNA synthesis occurs at a time later than viral DNA synthesis, and is also dependent upon it. Besides inhibitors of DNA synthesis, actinomycin D at certain concentrations appears to allow the accumulation of relatively large amounts of early RNA with concomitant suppression of late RNA synthesis (5). We have studied the sedimentation properties of viral RNA synthesized early in the infectious cycle, and of viral RNA made in the presence of cytosine arabinoside (an inhibitor of DNA synthesis) or in the presence of actinomycin (an inhibitor of RNA synthesis).

Viral DNA synthesis is undetectable before the 16th hr postinfection (2). In Fig. 4, we compare the sedimentation of late RNA (labeled from 40 to 46 hr postinfection, Fig. 4a) with early RNA (labeled from 2 to 16 hr postinfection, Fig. 4b). Although the 16*S* peak predominated later in infection, the 19*S* RNA appeared as the main species early in infection. Experiments with metabolic inhibitors produced similar results.

The effect of cytosine arabinoside on viral RNA is shown in Fig. 5. This drug almost completely suppressed the 16*S* species, but allowed accumulation of the 19*S* species. Other experiments, with the use of different hybridization techniques, demonstrated that under certain conditions 19*S* RNA is the sole viral RNA made in the presence of cytosine arabinoside (R. Weinberg, Z. Ben-Ishai, R. Dulbecco, *in preparation*). Actinomycin D, which allows accumulation of early RNA (5), enhanced the 19*S* RNA relative to the 16*S* RNA species. None of these experiments, however, demonstrated that the early 19*S* RNA was identical to the 19*S* RNA species observed late in the lytic cycle.

The formamide hybridization technique can be used to prepare relatively pure early RNA in spite of its low concentration in the cell. Table 2 shows that almost 3% of the radioactively labeled RNA present 40 to 46 hr postinfection hybridized to SV40 DNA, about 100 times more than the amount hybridizing in the mock-infected control. Early RNA, labeled from 2 to 16 hr postinfection, hybridized to a value only about four times the background amount. Early RNA could be further purified, however, by eluting this RNA and rehybridizing it in a second cycle of hybridization to SV40 DNA. Under these conditions, the early RNA hybridized to SV40 DNA to a value 30 times higher than to the blank filter, and the late RNA hybridized 300 times more to SV40 DNA than to a blank filter.

Heterogeneous nuclear RNA. The sedimentation analysis of extracts of unfractionated, infected cells indicated that SV40 RNA contains both homogeneous RNA species, and high-molecular-weight heterogeneous RNA (Fig. 1). Figure 6

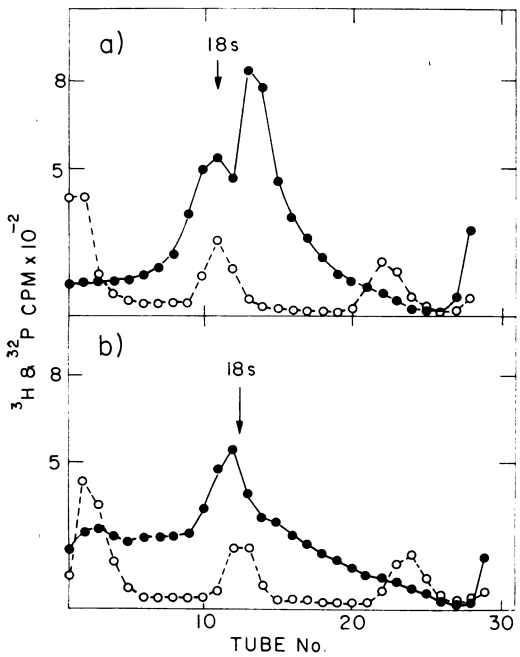

FIG. 4. *Sedimentation of viral RNA labeled early in the lytic cycle. Thirty-nine BS-C-1 cultures were infected with plaque-purified virus at 500 plaque-forming units/cell. A similar group of 39 cultures was mock-infected. Both groups were labeled with ^3H-uridine (250 μCi/ml, 30 Ci/mmole) from 2 to 16 hr postinfection. Control "late" RNA was extracted from two plates infected as above but labeled from 40 to 46 hr postinfection. ^3H-RNA was extracted from the infected cells, and the viral RNA was selected by formamide hybridization to plaque-purified SV40 DNA (Table 2, first cycle hybridization). The viral RNA was then analyzed on an SDS-sucrose gradient (30,000 rev/min, 16 hr, SW41 rotor). (a) Late RNA: 40 to 46 hr postinfection. (b) Early RNA: 2 to 16 hr postinfection. Symbols: ●, ^3H-viral RNA; ○, ^{32}P-ribosomal RNA marker.*

shows that, late in infection, the high-molecular-weight heterogeneous viral RNA is largely nuclear whereas the homogeneous species are largely cytoplasmic (21, 24). The gradients were centrifuged for a short time to demonstrate the large size of the heterogeneous material, and thus do not adequately resolve the two cytoplasmic species (Fig. 6b).

Figure 6 suggests that the more rapidly sedimenting nuclear RNA does not represent aggregation of low-molecular-weight RNA, because such material is present only in the nuclear fraction. However, a more rigorous proof of the covalent integrity of the high-molecular-weight RNA is provided by the DMSO gradient analysis of Fig. 7. Whole-cell RNA (nuclear plus cytoplasmic) from cells late in the lytic cycle was centrifuged on a DMSO gradient stabilized with sucrose. Although the DMSO gradient has low resolving power, and does not separate the two homogeneous cytoplasmic species, it demonstrates that much of the heterogeneous viral RNA sediments

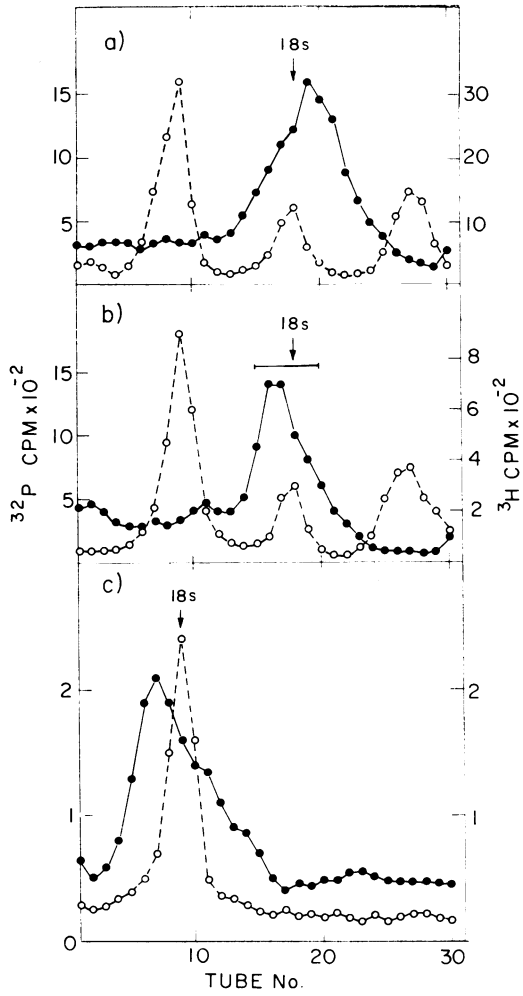

FIG. 5. *Effect of cytosine arabinoside on viral RNA. Three cultures of BS-C-1 cells were infected at a multiplicity of 100 plaque-forming units/cell. After virus adsorption, two plates were refed with medium containing cytosine arabinoside (AraC) at 5 μg/ml; the third was refed with normal medium. Cells were labeled in the presence of the drug from 22 to 33 hr postinfection in the case of the first two plates. The third was labeled at the same time without the drug. The RNA from the drug-treated and control cultures were hybridized in formamide to SV40 DNA, eluted, and analyzed on SDS-sucrose gradients. (a) Viral RNA from untreated control culture; (b) viral RNA from cytosine arabinoside-treated culture; (c) recentrifugation of fractions 15 to 20, panel b. Panels a and b: SDS-sucrose gradient, 25,000 rev/min, 16 hr, in an SW36 rotor. Panel c: SDS-sucrose gradient at 32,000 rev/min, 16 hr, in an SW36 rotor. Symbols: ●, ^3H-viral RNA; ○, ^{32}P-ribosomal RNA marker.*

TABLE 2. *Selection of virus-specific RNA labeled early in the lytic cycle*[a]

Origin of ^{3}H-RNA	Counts/min of input to hybridization	Percentage of input hybridized and eluted	
		SV40 DNA (10 μg)	Blank
First cycle of formamide hybridization			
Infected cells 2–16 hr pi[b]	1.5×10^9	0.120	0.028
Mock-infected cells	1.4×10^9	0.035	0.025
Infected cells 40–46 hr pi	7.0×10^7	2.85	0.030
Second cycle of formamide hybridization			
Infected cells 2–16 hr pi	9.0×10^5	4.40	0.150
Infected cells 40–46 hr pi	1.6×10^6	12.50	0.040

[a] The preparation of the RNA is described in the legend to Fig. 4. The eluates of the 1st cycle of hybridization in formamide were rehybridized in formamide in a second cycle of hybridization.

[b] Postinfection.

much further than even 28*S* ribosomal RNA. Since a complete transcript of the viral genome should sediment as 27*S* RNA, we can confirm the reports of others that there are RNA molecules of double and triple the length of the genome which contain viral sequences and which do not appear to be aggregates (1, 24).

The large viral homologous RNA molecules might derive from the transit of the RNA polymerase molecule several times around the circular viral DNA template before the nascent RNA chain is terminated. This possibility is unlikely in view of the data presented in Table 3. High-molecular-weight viral RNA was prepared by the formamide hybridization-elution procedure and sedimented on a DMSO gradient. The material sedimenting faster than 28*S* ribosomal RNA (Fig. 7a) hybridized back to SV40 DNA at a much lower efficiency than does viral homologous RNA smaller than 28*S* (Table 3). This lower efficiency of hybridization back to viral DNA may arise because high-molecular-weight viral RNA might hybridize with a lower efficiency than lower-molecular-weight viral RNA containing the same sequences. However, when the high-molecular-weight viral RNA was degraded by limited alkali treatment, the hybridization efficiency of the resulting fragments (Fig. 7b) did not increase (Table 3). This experiment indicates that the high-molecular-weight viral homologous RNA molecules prepared by the formamide hybridization-elution procedure contain a far smaller proportion of SV40 sequences than their lower-molecular-weight counterparts (see also 8, 24).

Additional control experiments (S. O. Warnaar, *unpublished data*) indicated that the high-molecular-weight viral RNA is not an artifact of the type of virus used to infect the cells, nor of the type of viral DNA used for the hybridization-elution step. Thus, this viral RNA can be isolated from cells infected with plaque-purified SV40 virus, by hybridization to DNA derived from plaque-purified virus. The latter control precludes the possibility that the nonviral sequences found in the heterogeneous RNA arise exclusively from host DNA sequences incorporated into the viral DNA of SV40 virus passaged at high titer (11). Recent experiments, with the formamide hybridization-elution technique described in this

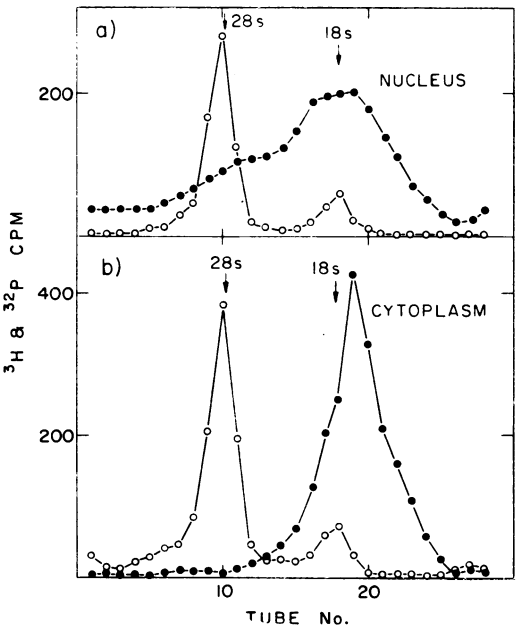

FIG. 6. *Nuclear and cytoplasmic virus-specific RNA. One infected BS-C-1 culture was labeled as in Fig. 1. After washing with phosphate-buffered saline, the cells were swollen in RSB buffer (18). The cells were homogenized with a Dounce-type homogenizer and the nuclei were sedimented by centrifugation. The supernatant fluid was termed the cytoplasmic fraction; the pellet was termed the nuclear fraction. RNA from each fraction was extracted and hybridized in formamide to SV40 DNA. The eluted RNA was sedimented as in Fig. 1 but for 14 hr. (a) Viral nuclear RNA; (b) viral cytoplasmic RNA. Symbols: ●, ^{3}H-viral RNA; ○, ^{32}P-ribosomal RNA marker.*

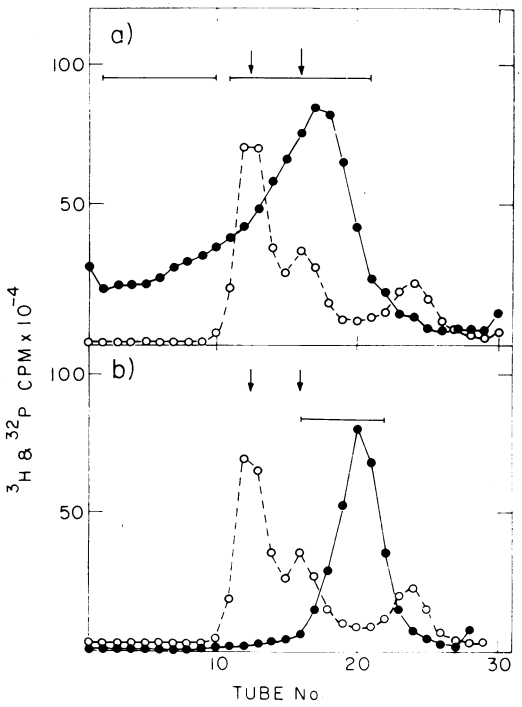

FIG. 7. *DMSO gradient analysis of high-molecular-weight viral RNA. Five cultures of SV40-infected BS-C-1 cells were labeled with ^3H-uridine (200 μCi/ml, 30 Ci/mmole) from 24 to 47 hr postinfection. (a) ^3H-RNA was extracted from whole cells, hybridized to SV40 DNA in formamide, eluted, and centrifuged through a 5 to 20% sucrose gradient in 99% DMSO. The input to the formamide hybridization was 3.5 × 10^8 counts/min; 5.5 × 10^6 counts/min were eluted from the SV40 filter, and 3.3 × 10^3 counts/min were eluted from a blank filter. The eluted RNA was centrifuged at 45,000 rev/min for 20 hr at 26 C in an SW50.1 rotor. (b) Fractions 2 to 10 of panel a were pooled; one portion of the pooled RNA was treated with 0.05 M Na_2CO_3 (pH 11.0) for 3 min at 80 C, cooled, neutralized, and recentrifuged as above. Symbols: ●, ^3H-viral RNA; ○, ^{32}P-ribosomal RNA marker (the arrows denote the positions of the 18S and 28S species of ribosomal RNA).*

paper, have demonstrated that high-molecular-weight viral RNA molecules from lytically infected cells contain covalently linked sequences which are complementary to both the viral and host-cell genomes (S. Rozenblatt and E. Winocour, *in preparation*).

DISCUSSION

The application of the formamide hybridization technique to SV40 RNA metabolism has indicated the existence of discrete viral RNA species. A 19S species is made early in infection, whereas 16S and 19S species are made late in the infectious cycle. The existence of one or both of these species was suggested by previous reports (15, 24). These homogeneous species all appear to be localized in the cytoplasm, and all contain polyadenylic acid sequences (R. Weinberg, Z. Ben-Ishai, J. E. Newbold, and R. Dulbecco, *in preparation*). They are probably messenger RNA species, but this can be shown only by a demonstration of their functional association with polyribosomes.

It would be tempting to suggest that the early 19S species and the late 19S species are identical in base sequence and represent the early portion of the viral genome, whereas the 16S RNA is transcribed from the late portion of the genome. The molecular weights of the 16S and 19S species (ca. 650,000 and 900,000) together add up to approximately the molecular weight expected for a complete transcript of the viral genome (ca. 1.5 × 10^6). However, preliminary experiments show that the late 16S and 19S species saturate similar proportions of the viral genome, and appear to compete strongly with each other for the same DNA sequences (S. O. Warnaar and E. Winocour, *unpublished data*). The late 16S and 19S species may thus be derived from overlapping parts of the viral genome rather than mutually exclusive portions, and the 19S and 16S may have a precursor-product relationship. The heterogeneous high-molecular-weight

TABLE 3. *Rehybridization of large virus-specific RNA to SV40 DNA before and after alkali degradation to smaller size*

Origin of ^3H-RNA[a]	^3H input (counts/min)	Percentage of input bound to filters	
		SV40 DNA (10 μg)	Blank
>28S RNA[b]	33,000	7.7	1.00
>28S RNA after alkali degradation to 10–18S RNA[c]	60,600 30,300	8.9 8.8	0.25 0.30
<28S RNA[d]	13,000	30.0	1.20

[a] The preparation and fractionation of this RNA on a DMSO-sucrose gradient is described in the legend to Fig. 7.

[b] Pooled fractions 2–10 of Fig. 7a after deoxyribonuclease treatment.

[c] A sample of pooled fractions 2–10 of Fig. 7a was treated with 0.05 M Na_2CO_3 (pH 11.0) for 3 min at 80 C. After cooling and neutralization, the RNA was centrifuged on a DMSO gradient as shown in Fig. 7b. Fractions 16–22 of this second gradient were pooled and used, after deoxyribonuclease treatment.

[d] Fractions 11–21 in Fig. 7a after deoxyribonuclease treatment.

nuclear RNA of the virus may, in turn, be precursor to the cytoplasmic homogeneous species.

SV40-transformed cells contain a virus-related high-molecular-weight nuclear RNA (12, 24) which contains sequences complementary to both the viral and cellular genomes (25). Such molecules arise, presumably, by the co-transcription of integrated viral DNA and adjacent cellular DNA. It has recently been demonstrated that, under certain conditions of lytic infection, closed circular SV40 DNA molecules containing a covalently linked segment of host DNA are produced (11, 23). These molecules probably arise from virus DNA which integrated into host chromosomal DNA and which was subsequently excised together with a segment of host DNA. Recent experiments have also demonstrated that SV40-induced high-molecular-weight nuclear RNA, from lytically infected cells, hybridizes both to plaque-purified viral DNA and to host-cell DNA (S. Rozenblatt and E. Winocour, *in preparation*). We would therefore suggest that the high molecular weight of some of the SV40 nuclear RNA is due to the co-transcription of host and viral DNA sequences during lytic infection.

The formamide hybridization technique presented here can be used to make viral RNA for analytical and preparative purposes. The technique may prove useful for the isolation of other RNA species transcribed from episomal or viral genomes. We are presently attempting in vitro translation of the viral RNA prepared by this procedure.

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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. Nat. Acad. Sci. U.S.A. 68:2231–2235.
2. Aloni, Y., E. Winocour, and L. Sachs. 1968. Characterization of the simian virus 40-specific RNA in virus yielding and transformed cells. J. Mol. Biol. 31:415–429.
3. Black, P. H., E. M. Crawford, and L. V. Crawford. 1964. The purification of simian virus 40. Virology 24:381–387.
4. Butel, J. S., and F. Rapp. 1965. The effect of arabino-furanosylcytosine on the growth cycle of simian virus 40. Virology 27:490–495.
5. Carp, R. I., G. Sauer, and F. Sokol. 1969. The effect of actinomycin D on the transcription and replication of simian virus 40 deoxyribonucleic acid. Virology 37:214–226.
6. Fry, M., and M. Artman. 1969. Deoxyribonucleic acid-ribonucleic acid hybridization. Biochem. J. 115:287–294.
7. Gillespie, D., and S. Spiegelman. 1965. A quantitative assay for DNA-RNA hybrids with DNA immobilized as a membrane. J. Mol. Biol. 12:829–842.
8. Jaenisch, R. 1972. Evidence for SV40 specific RNA containing virus and host specific sequences. Nature N. Biol. 235: 46–47.
9. Katz, L., and S. Penman. 1966. The solvent denaturation of double-stranded RNA from poliovirus infected HeLa cells. Biochem. Biophys. Res. Commun. 23:557–560.
10. Kit, S., D. R. Dubbs, P. M. Frearson, and J. L. Melnick. 1966. Enzyme induction in SV40 infected green monkey kidney cultures. Virology 29:69–83.
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. Lindberg, U., and J. E. Darnell. 1970. SV40-specific RNA in the nucleus and polyribosomes of transformed cells. Proc. Nat. Acad. Sci. U.S.A. 65:1089–1096.
13. McConaughy, B. L., C. D. Laird, and B. J. McCarthy. 1969. Nucleic acid reassociation in formamide. Biochemistry 8:3289–3295.
14. Martin, M. A., and D. Axelrod. 1969. SV40 gene activity during lytic infection and in a series of SV40 transformed mouse cells. Proc. Nat. Acad. Sci. U.S.A. 64:1203–1210.
15. Martin, M. A., and J. C. Byrne. 1970. Sedimentation properties of simian virus 40-specific ribonucleic acid present in green monkey cells during productive infection and in mouse cells undergoing abortive infection. J. Virol. 6:463–469.
16. Oda, K., and R. Dulbecco. 1968. Induction of cellular RNA synthesis in BS-C-1 cells infected by SV40. Virology 35:439–444.
17. Oda, K., and R. Dulbecco. 1968. Regulation of transcription of the SV40 DNA in productively infected and in transformed cells. Proc. Nat. Acad. Sci. U.S.A. 60:525–532.
18. Penman, S. 1966. RNA metabolism in the HeLa cell nucleus. J. Mol. Biol. 17:117–130.
19. Sauer, G., and J. R. Kidwai. 1968. The transcription of the SV40 genome in productively infected and transformed cells. Proc. Nat. Acad. Sci. U.S.A. 61:1256–1263.
20. Sauer, G. 1971. Apparent differences in transcriptional control in cells productively infected and transformed by SV40. Nature N. Biol. 231:135–138.
21. Sokol, F., and R. I. Carp. 1971. Molecular size of simian virus 40-specific RNA synthesized in productively infected cells. J. Gen. Virol. 11:177–188.
22. Strauss, J. H., Jr., R. B. Kelly, and R. L. Sinsheimer. 1968. Denaturation of RNA with dimethylsulfoxide. Biopolymers 6:793–807.
23. Tai, H. T., C. A. Smith, P. A. Sharp, and J. Vinograd. 1972. Sequence heterogeneity in closed simian virus 40 deoxyribonucleic acid. J. Virol. 9:317–325.
24. Tonegawa, S., G. Walter, A. Bernardini, and R. Dulbecco. 1970. Transcription of the SV40 genome in transformed cells and during lytic infection. Cold Spring Harbor Symp. Quant. Biol. 35:823–831.
25. 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 N. Biol. 232:73–76.
26. Weinberg, R., M. Willems, U. Loening, and S. Penman. 1967. Acrylamide gel electrophoresis of HeLa cell nucleolar RNA. Proc. Nat. Acad. Sci. U.S.A. 58:1088–1095.