Sedimentation Properties of Simian Virus 40-Specific Ribonucleic Acid Present in Green Monkey Cells During Productive Infection and in Mouse Cells Undergoing Abortive Infection

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Received for publication 23 June 1970

The size distribution of polyribosome-associated simian virus 40 (SV40) ribonucleic acid (RNA) was examined at various times after productive infection. Eight hours after infection, virus-specific RNA was detected in the 14 to 17S region of a sucrose gradient by deoxyribonucleic acid (DNA)-RNA hybridization; RNA present in fractions sedimenting more rapidly did not react with SV40 DNA. At successively later times, SV40 RNA was detected in more rapidly sedimenting regions. By 24 hr, a portion of the SV40 RNA was detected in the 28S region, sedimenting slightly more rapidly than a MS2 RNA marker. Nuclear SV40 RNA, prepared from cells 48 hr after infection, was distributed in more rapidly sedimenting regions of the gradient, peaking at about 32 to 34S. Some nuclear virus-specific RNA could be detected in the 45 to 50S region. During the abortive infection of mouse cells, the sedimentation profile of SV40 RNA was very similar to that observed during the early phases of the lytic cycle.

Simian virus 40 (SV40)-specific ribonucleic acid (RNA) has been detected in green monkey cells undergoing lytic infection (1) as well as in cells transformed by this agent (1, 11, 15). When total cellular RNA is used to monitor SV40 gene activity during productive infection, it has been shown that 50% of the viral genome is transcribed (10). No attempt was made at that time to determine whether this RNA was polycistronic or consisted of a series of gene products, the sum of which was separately transcribed from one SV40 deoxyribonucleic acid (DNA) strand. It has been previously shown that during polyoma infection 50% of the viral DNA is transcribed (9), and virus-specific RNA is present with sedimentation properties consistent with polycistronic transcription of one DNA strand— 1.6×10^6 daltons (5). In both cases, DNA transcription was monitored with radiolabeled total cellular RNA.

Virus-specific RNA associated with cytoplasmic polyribosomes is the RNA form which is actively involved in the synthesis of the proteins necessary for productive infection. For this reason, we decided to examine this species of cytoplasmic SV40 RNA at various times during the lytic cycle. Our results indicate that early in infection only slowly sedimenting RNA forms are present. Later, progressively larger SV40-specific RNA molecules were observed, a portion of

which had sedimentation properties compatible with the transcription of one SV40 strand.

MATERIALS AND METHODS

Preparation of SV40 DNA. Confluent monolayers of Vero (3) cells, growing in 1-gal roller bottles, were infected with small-plaque SV40 virus at a multiplicity of 0.25 to 0.50 plaque-forming units (PFU)/cell. Cells were generally harvested 6 to 7 days after infection, and the virus was purified as previously described (10). SV40 DNA was released by exposing purified virus preparations to 1% sodium dodecyl sulfate (SDS) and 0.01 M ethylenediaminetetraacetic acid (EDTA), pH 7.5, at 50 C for 30 min. The SDS was removed by precipitation with CsCl (1 g/ml; 18), and the DNA was centrifuged in a no. 40 fixed-angle Spinco rotor for 60 hr at 38,000 rev/min at 5 C, in the presence of ethidium bromide [200 μ g/ml (14)]. SV40 DNA I was collected with a Pasteur pipette, and the ethidium bromide was removed by chromatography on Dowex-50 equilibrated with one-tenth standard saline citrate (SSC). The DNA was further purified by gel filtration on G-100 Sephadex equilibrated with 0.1 M NaCl.

Preparation of 32 P-labeled polyribosomes from cells productively infected with SV40 virus. Confluent monolayers of Vero cells were exposed to small-plaque SV40 virus at a multiplicity of 25 to 50 PFU/cell. Virus was allowed to adsorb for 2 hr at 37 C. Phosphate-free Eagle's basal no. 2 medium was added to the cells 16 hr prior to the addition of 32 P-orthophosphate.

In one case (polyribosomes prepared from cells 8 hr after infection), the phosphate-free media was added 8 hr prior to infection. The infected cells were labeled for 75 min in 50 ml of fresh phosphate-free media containing 50 μ Ci of carrier-free 32 P-orthophosphate per ml. The cells were then harvested with 0.25% trypsin, washed twice in phosphate-buffered saline and once with 0.01 M tris(hydroxymethyl)aminomethane (Tris), pH 7.4, 0.01 M NaCl, and 0.0015 M $MgCl_2$ [RSB (12)]. The infected Vero cells were allowed to swell in RSB for 5 min at 0 C and lysed by Dounce homogenization (12 strokes). Nuclei were removed by sedimentation at 5,000 rev/min for 10 min. Sodium deoxycholate (0.5%), Brij 58 (0.5%; Atlas Chemical Industries, Inc., Wilmington, Del.), and $MgCl_2$ (0.07 M) were added, and the cytoplasmic fraction was kept in an ice-water bath for at least 90 min (6). Polyribosomes were pelleted at $17,000 \times g$ for 10 min, resuspended in RSB, layered on 10 to 34% (w/v) sucrose gradients containing RSB, and centrifuged for 90 min at 23,000 rev/min at 5 C in a SW 25.1 rotor. Fractions were collected by puncturing the bottom of the tube, and the polyribosomes were pooled as indicated.

Preparation of 32 P-labeled polyribosomes from uninfected Vero cells. Vero cells (200,000 cells/ml) were added to 1-gal roller bottles containing Eagle's basal no. 2 medium (400 ml) supplemented with 10% fetal bovine serum. When the cells had achieved approximately 75% of their final density, the medium was changed to phosphate-free Eagle's basal no. 2. After 16 hr, the cells were exposed to carrier-free 32 P-orthophosphate (50 μ Ci/ml) in 50 ml of the phosphate-free media. Cells were harvested 75 min later, and polyribosomes were prepared as described above.

Preparation of polyribosome-associated RNA. Polyribosomes were pooled and precipitated with 2.5 volumes of 95% ethanol. The polyribosome pellet was resuspended in 0.1 M NaCl, 0.01 M Tris (pH 7.5), and 0.001 M EDTA and exposed to 0.5% SDS for 2 hr. The dissociated polyribosomes were layered on a 15 to 30% (w/v) sucrose gradient containing 0.1 M NaCl, 0.01 M Tris (pH 7.5), 0.01 M EDTA, and 0.5% SDS (SDS buffer) and centrifuged for 14 hr at 21,000 rev/min at 20 C. Exposure of the dissociated polyribosomes to 60 C for 5 min prior to centrifugation did not affect the sedimentation profile observed. Fractions from the gradient were pooled and digested sequentially with 25 μ g of electrophoretically purified pancreatic deoxyribonuclease (Worthington Biochemical Corp.) per ml and 50 μ g of self-digested Pronase (Calbiochem) per ml, each for 1 hr at room temperature. The pooled fractions were then extracted with phenol, ethanol-precipitated in the presence of yeast carrier RNA (50 μ g/ml), and resuspended in 0.2 to 0.3 ml of one-tenth strength SSC.

Preparation of nuclear RNA from infected Vero cells. A confluent monolayer of Vero cells growing in a 1-gal bottle was infected with small-plaque SV40 at a multiplicity of 25 to 50 PFU/cell. Forty-eight hours after infection, the cells were exposed to 1.0 mCi of 3 H-uridine (21 Ci/mmol) in 50 ml of Eagle's basal no. 2 medium for 75 min. Cells were harvested and fractionated as described above. The nuclear pellet was washed with phosphate-buffered saline, resuspended

in 0.1 M NaCl, 0.01 M sodium acetate, 0.005 M $MgCl_2$, and 0.5% SDS and digested with deoxyribonuclease (100 μ g/ml) for 1 hr at room temperature. After a phenol extraction at 60 C, the preparation was precipitated with 2 volumes of 95% ethanol, resuspended in SDS buffer, and centrifuged as described above.

DNA-RNA hybridization. Small-plaque SV40 DNA I was heated in $1 \times$ SSC for 15 min and immobilized on 50-mm nitrocellulose filters (type B6, Schleicher & Schuell Co., Keene, N.H.; reference 4). Nitrocellulose filters (7 mm), containing about 0.3 μ g of SV40 DNA, were added to reaction mixtures containing 0.8 M NaCl, 0.001 M *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES; Calbiochem, Los Angeles, Calif.), pH 7.5, 0.1% SDS, and labeled RNA in a volume of 0.25 ml. After a 16-hr incubation at 68 C, the filters were exposed to pancreatic ribonuclease (20 μ g/ml) in $2 \times$ SSC for 1 hr, extensively washed, dried, and counted.

RESULTS

Sedimentation properties of SV40 RNA present late in infection. To demonstrate that the equivalent of one strand of SV40 DNA is transcribed during lytic infection (10), it was necessary to prepare and pool RNA species made at various times during the lytic cycle. The asynchronous nature of SV40 infection allowed preparation of labeled polyribosomes at a time (48 hr after infection) when cells at various stages of infection were certain to be present. Polyribosome-associated messenger RNA was isolated after treatment with 0.5% SDS and sucrose gradient centrifugation. Fractions were pooled, purified as described above, and added to DNA-RNA hybridization reaction mixtures. Figure 1 shows the sedimentation properties of SV40-specific RNA prepared from cells 48 hr after infection. It can be seen that, although SV40 messenger RNA is very heterogeneous in molecular size, a significant proportion sediments in the region of 28S ribosomal RNA. The more rapidly sedimenting SV40-specific RNA molecules appear to migrate slightly faster in the gradient than the marker MS2 RNA [1.1×10^6 daltons (16)].

Size distribution of SV40 RNA at various times during infection. At 8, 16, 24, and 48 hr after infection with SV40, Vero cells were exposed to 32 P-orthophosphate (50 μ Ci/ml) for 75 min. The polyribosome pattern observed at each of these times is shown in Fig. 2. It should be mentioned that polyribosomes are virtually undetectable in *confluent uninfected* Vero cells. After infection, however, polyribosomes were readily detected, reaching a peak at 16 hr and gradually declining over the next 32 hr. In each case, polyribosomes were prepared from 2×10^8 -infected Vero cells.

The RNA associated with the 8-, 16-, and 24-hr polyribosome preparations was purified as des-

FIG. 1. Size distribution of SV40-specific RNA 48 hr after infection. ^{32}P -labeled polyribosomes, prepared from Vero cells 48 hr after infection, were mixed with H^3 MS2 RNA and sedimented through a 15 to 30% (w/v) sucrose gradient containing SDS buffer. Fractions were collected by puncturing the bottom of the centrifuge tube and were checked for absorbance at 260 nm. Samples of each fraction were precipitated with 5% trichloroacetic acid to determine the position of the MS2 marker (○). The fractions were pooled in groups of three, purified as described in the text, and assayed for the presence of SV40 RNA (●) by DNA-RNA hybridization.

cribed above. It can be seen from Fig. 3 that SV40 RNA of increasing size appears during the course of productive infection. At the earliest time (8 hr), a portion of the SV40 gene product sediments in the 15 to 17S region of the gradient (Fig. 3A). RNA present in fractions which sedimented more rapidly did not form stable duplex molecules with SV40 DNA. Eight hours later (16 hr after infection) SV40 messenger RNA was detected in the 22 to 25S region of the gradient (Fig. 3B). By 24 hr, a portion of the virus-specific RNA was measurable in the 28S ribosomal RNA region (Fig. 3C). The size distribution of the polyribosome-associated SV40 RNA observed 24 hr after infection is very similar to that detected at 48 hr (Fig. 1). These experiments suggest that during the course of lytic infection the size distribution of cytoplasmic SV40 RNA molecules gradually increases. Shortly after the time when viral DNA synthesis occurs (24 hr), SV40 RNA detected in the region of the 28S ribosomal RNA [1.6×10^6 to 1.9×10^6 daltons (8, 13)] may represent the complete transcription of a poly-

cistronic message from one strand of SV40 DNA [1.5×10^6 daltons (2)].

Figure 4 shows that SV40-specific RNA could not be detected in uninfected Vero cells. Actively growing cells instead of confluent monolayers were used in this experiment because of the low level of polyribosomes present.

Resedimentation of SV40-specific RNA. To rule out the possibility that the SV40 RNA detected in the 28S region did not represent small RNA molecules that had diffused into the more rapidly sedimenting regions of the gradient, the experiment shown in Fig. 5 was carried out. Polyribosomes, labeled 48 hr after infection with ^{32}P -orthophosphate, were prepared as described

FIG. 2. Sedimentation profiles of polyribosomes prepared from SV40-infected Vero cells. Vero cells, growing in 1-gal roller bottles, were harvested at 8, 16, 24, and 48 hr after infection with SV40, after a 75-min exposure to ^{32}P -orthophosphate. In each case, approximately 2×10^8 cells were harvested, and polyribosomes were prepared as described in the text. The polyribosomes were sedimented through a 10 to 34% (w/v) sucrose gradient containing RSB, as described in the text, and fractions were collected by puncturing the bottom of the centrifuge tubes. Under the centrifugation conditions employed, the 74S monosome peak was located in the region of fractions 48-50. Polyribosomes were pooled as follows: 8 hr after infection, fractions 13-40; 16 hr after infection, fractions 5-39; 24 hr after infection, fractions 12-39; 48 hr after infection, fractions 13-39.

FIG. 3. Size distribution of SV40-specific RNA at 8, 16, and 24 hr after infection. Polyribosome-associated RNA was prepared from cells 8 hr (A), 16 hr (B), and 24 hr (C) after infection with SV40 as described in the text. Fractions from 15 to 30% (w/v) sucrose gradients containing SDS buffer were analyzed for SV40 RNA (●) by DNA-RNA hybridization.

FIG. 4. Assay of SV40 RNA in uninfected Vero cells. ^{32}P -polyribosomes were prepared from uninfected Vero cells that were 80% confluent (A). Polyribosomes were dissociated as described in the text and fractions were tested for SV40 RNA (○) by DNA-RNA hybridization (B).

above (Fig. 5A). The polyribosome-associated RNA sedimenting in the regions of the 28S or 18S ribosomal RNA species (Fig. 5B) were separately collected and resedimented through sucrose. Figure 5C shows the results of this experiment and indicates that the high-molecular-weight fraction of SV40-specific RNA is distributed throughout the region of the 28S ribosomal RNA. This result suggests that, although SV40 RNA is heterogeneous in size, the high-molecular-weight virus-specific RNA sedimenting with the 28S ribosomal RNA does not merely reflect the diffusion of smaller RNA species from more slowly sedimenting regions of the gradient.

The SV40 RNA sedimenting with the 28S ribosomal RNA was never heated or purposely sheared prior to its addition to the DNA-RNA hybridization mixture. Therefore, the possibility exists that these RNA molecules may contain

FIG. 5. Resedimentation of infected Vero cell RNA. (A) Zonal sedimentation profile of ^{32}P -polyribosomes prepared from Vero cells 48 hr after infection with SV40 virus. (B) Fractions 4-38 from the sucrose gradient shown in (A) were pooled and preparatively sedimented through a 15 to 30% (w/v) sucrose gradient containing SDS buffer as described in the text. (C) Fractions 30-32 and 44-46 from the sucrose gradient shown in (B) were separately pooled, purified as described in the text, and resedimented through a 15 to 30% (w/v) sucrose gradient containing SDS buffer. Fractions of RNA originally sedimenting in the region of 28S (●) or 18S (○) ribosomal RNA were analyzed for absorption (solid line) or virus-specific RNA (broken line) as previously described.

covalently linked green monkey sequences. If such host sequences were "sandwiched" between SV40 sequences on the same RNA molecules, the resulting SV40 DNA-SV40 RNA duplex molecules formed might still possess the necessary stability to withstand the action of pancreatic ribonuclease. This question was answered by recovering the ^{32}P -RNA that had reacted with immobilized SV40 DNA and incubating it with nitrocellulose filters containing SV40 and green monkey DNA molecules. Table 1 demonstrates that the labeled SV40 RNA molecules sedimenting in the region of the 28S ribosomal RNA contain no host sequences.

Size distribution of nuclear SV40 RNA. Since polycistronic SV40 RNA approaching the molecular size of one strand of SV40 DNA could be detected in the cytoplasm of infected Vero cells, we decided to characterize the virus-specific RNA present in the nuclei of these cells. Vero cells were labeled with ^3H -uridine (20 $\mu\text{Ci}/\text{ml}$) for 75 min 48 hr after infection with SV40. Nuclear RNA was prepared as described above and analyzed for virus-specific RNA after sedimentation in a 15 to 30% (w/v) sucrose gradient containing SDS buffer. Unlabeled 28 and 18S ribosomal RNA was simultaneously sedimented in a separate tube. In contrast with the results observed earlier (Fig. 1, 3) with cytoplasmic RNA, nuclear SV40 RNA was distributed in the more rapidly sedimenting regions of the gradient, peaking at about 32 to 34S (Fig. 6). Some virus-specific RNA could be detected in the 45 to 50S region

suggesting that molecules larger than unit length may be transcribed in the nuclei of these cells.

Sedimentation properties of SV40 RNA during abortive infection. Replication of SV40 does not occur in mouse cells (nonpermissive). Some SV40 nonstructural proteins (e.g., T antigen) associated with the "early" period of lytic infection can be detected after infection of these cells, but so called "late" viral functions, such as DNA replication and the synthesis of capsid proteins, are inhibited. Since some of the initial biochemical events associated with transformation occur during abortive infection, experiments characterizing the SV40 RNA present in such mouse cells were carried out. A confluent monolayer of mouse cells [AL/N (17)] was infected with SV40 at a multiplicity of 400 PFU/cell. The infected cells were labeled for 2 hr with ^{32}P -orthophosphate 68 hr after infection, and polyribosomes were prepared as previously described. Figure 7 shows that a portion of the SV40 RNA sedimented in the region of the 18S ribosomal RNA, but none of the more rapidly sedimenting virus-specific RNA

TABLE 1. *Rehybridization of the simian virus 40 (SV40) RNA sedimenting in the region of 28S ribosomal RNA^a*

DNA filter	RNA reacting
	%
SV40.....	34.8
Green monkey.....	0.0
Blank.....	0.2

^a Polyribosome-associated RNA present in Vero cells 48 hr after infection and sedimenting in the region of 28S ribosomal RNA was prepared as shown in Fig. 5. This RNA (180,000 counts/min) was incubated with a nitrocellulose filter containing 8 μg of SV40 DNA. The filter was extensively washed at 68 C in 4X SSC, and the virus-specific RNA (532 counts/min) was eluted after two 10-min exposures to 0.01 M EDTA and 0.1% SDS at 95 C. The recovered SV40 RNA was incubated with three 7-mm nitrocellulose filters containing 0.3 μg of SV40 DNA, 16 μg of green monkey DNA, and no DNA. After 16 hr of incubation at 68 C, the filters were treated with ribonuclease and assayed for radioactivity as described in Fig. 2.

FIG. 6. *Size distribution of SV40 RNA in the nuclei of infected Vero cells. Nuclei, labeled with ^3H -uridine, were prepared from Vero cells 48 hr after infection with SV40 as described in the text. The nuclear RNA was sedimented through a 15 to 30% (w/v) sucrose gradient containing SDS buffer, and fractions were analyzed for adsorption at 260 nm and SV40 RNA. Purified 28 and 18S ribosomal RNA was centrifuged in a separate tube and used as markers. SV40 RNA (●) was detected by DNA-RNA hybridization.*

FIG. 7. Sedimentation profile of the SV40 RNA present in mouse cells 68 hr after infection. A continuous line (AL/N) of mouse cells was infected with SV40 at a multiplicity of 400 PFU/cell and exposed for 90 min to ^{32}P -orthophosphate (200 $\mu\text{Ci}/\text{ml}$) 68 hr after infection. Polyribosome associated RNA was prepared as described in the text. Fractions from a 15 to 30% (w/v) sucrose gradient containing SDS buffer were analyzed for SV40 RNA (broken line) by DNA-RNA hybridization.

observed late in productive infection (Fig. 1) was detected. In fact, the size distribution of the SV40 RNA during abortive infection is quite similar to that seen during the early phases (Fig. 3A) of the lytic cycle.

DISCUSSION

It has previously been shown that during lytic infection 50% of SV40 DNA is transcribed (10). Those results, obtained from DNA-RNA saturation-hybridization experiments do not shed any light on the physical size of SV40-specific RNA. Recent work with polyoma virus suggests that RNA molecules are present during productive infection having molecular weights equivalent to one strand of circular polyoma DNA (5). Our results indicate that by 24 hr after infection the polyribosome-associated virus-specific RNA has a molecular size approaching one-half of the SV40 genome. Our data also show that the size distribution of SV40 RNA gradually increases during the course of productive infection. At 8 hr, for example, we were able to detect RNA molecules in

the 5×10^5 to 6×10^5 dalton range. Progressively larger SV40 RNA was seen at 16 and 24 hr after infection, with little further change at 48 hr.

Aloni et al. (1) have shown that "early" RNA is present throughout the infectious cycle. An additional species of SV40 RNA ("late") was also detected after the onset of viral DNA synthesis. The slowly sedimenting virus-specific RNA present at 8 hr (Fig. 3A) may represent those cistron(s) which are transcribed from DNA sequences coding for early functions. Late in infection, the pattern of SV40 DNA transcription is altered so that additional regions of the viral DNA are expressed, resulting in RNA molecules of increasing size (Fig. 3B, 3C). We are currently characterizing the slowly sedimenting RNA present early in infection and the high-molecular-weight species found at 24 and 48 hr to see if similar species of RNA are present in these two size classes.

Little is known about the physical properties of newly transcribed animal viral RNA. The SV40 RNA detected in the nuclei of infected cells sediments over a broad region of the sucrose gradient (Fig. 6). Some of the RNA is present in regions where molecules having molecular weights greater than 1.5×10^6 daltons would be expected to sediment. This result is similar to the findings of Lindberg and Darnell (7), who were able to detect virus-specific RNA in the nuclei of SV40-transformed cells which sediment more rapidly than 45S nuclear RNA. The presence of SV40 lytic RNA in the 45 to 50S region suggests that SV40 gene products may be transcribed in multiple units, resulting in molecules containing more than one equivalent of viral RNA. Such RNA molecules would have to be cleaved into smaller functional units prior to attachment to cytoplasmic ribosomes. We are presently characterizing the properties of nuclear SV40 RNA during productive and transforming infection.

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