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Characterization of Two Simian Virus 40-Specific RNA Molecules from Infected BS-C-1 Cells

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Two discrete simian virus 40 (SV40) RNA species sedimenting at 19 and 16*S*, respectively, that are present in infected BS-C-1 cells were characterized with respect to the base composition and the ribonuclease T1 fingerprints. The base composition of the 19*S* SV40 RNA was found to be cytidylic acid (C), 23.0; adenylic acid (A), 28.3; guanylic acid (G), 23.9; and uridylic acid (U), 24.8; that of the 16*S* SV40 RNA was C, 19.3; A, 34.0; G, 22.0; and U, 24.7 mol%. Analysis of the ribonuclease T1 fingerprints indicated a difference in the base sequence of the 19 and 16*S* SV40 RNA. The presence of long sequences of adenylic acid residues (poly A) in these viral RNAs was confirmed.

In a recent communication (15) the isolation of two simian virus 40 (SV40)-specific RNA molecules from lytically infected BS-C-1 cells, by use of a low-temperature hybridization technique in formamide, was reported. These two RNA species sedimented at 19 and 16*S*, respectively. In addition to these two species a heterogeneous faster sedimenting fraction which contained molecules having molecular weights of up to three times the value expected for a transcript of the whole SV40 genome was found. It was shown that the SV40 RNA sedimenting at 19*S* was present both early and late in the infection cycle, whereas the 16*S* RNA appeared only late after infection.

In this paper we report the base composition of 19 and 16*S* SV40 RNA isolated from infected cells late after infection and the base composition of 19*S* SV40 RNA synthesized in the presence of cytosine arabinoside.

RNase T1 fingerprints of SV40 RNA indicate a considerable difference in base sequence between 19 and 16*S* RNA.

We present additional evidence confirming the presence of long sequences of adenylic acid residues in these SV40-specific RNAs (14) of the type reported for a number of viral and mammalian mRNAs (4, 5, 8).

MATERIALS AND METHODS

Cells, medium, virus, and infection of cells. BS-C-1 cells originally obtained from Flow Laboratories were grown in 9-cm plastic Petri dishes in Eagle medium supplemented with 10% calf serum. SV40 strain 777 was obtained from E. Winocour. For infection of cells the medium was removed, and the cells were infected with 10 PFU/cell in 1 ml of medium. After an absorption period of 1 h at 37 C, fresh medium supplemented with 2% inactivated horse serum was added.

Labeling of cells and isolation of cytoplasmic RNA. The infected cells were labeled in 5 ml per plate of Eagle medium without phosphate; 0.1 mCi of ^{32}P-orthophosphate per ml was added during the interval indicated. A preparation of early 19*S* SV40 RNA was extracted from cells infected with SV40 virus in the presence of 5 μg of cytosine arabinoside (Ara C) per ml and labeled with ^{32}P from 10- to 30-h postinfection.

After labeling and washing with phosphate buffered saline the cells were lysed with 1% Nonidet P40 in phosphate buffered saline. The nuclei were centrifuged off and discarded. From the cytoplasmic extract, RNA was prepared by extensive shaking with 90% phenol and chloroform as previously described (15). After precipitating the RNA with two volumes of ethanol it was dissolved in hybridization buffer containing 50% (vol/vol) formamide, 0.75 M sodium chloride, 0.01 M Tris, and 0.5% sodium dodecyl sulfate (SDS), final pH 7.4.

Purification of SV40 RNA. The SV40-specific RNA was extracted from the total cytoplasmic RNA by hybridization to SV40 DNA on nitrocellulose filters in hybridization buffer at 37 C for 17 h. The filters were washed extensively, and the SV40 RNA was eluted from the filter with 90% formamide in water at pH 8.1. The SV40 RNA in the eluate was precipitated with two volumes of ethanol after the addition of carrier tRNA and sodium chloride. It was then dissolved in SDS buffer containing 0.01 M Tris, 0.001 M EDTA, 0.1 M sodium chloride, and 0.5% SDS, final pH 7.4. Linear sucrose gradients from 15 to 30%

(wt/wt) sucrose in SDS buffer were prepared in Spinco rotor SW 41 tubes. The RNA was layered on the gradient and spun at 20 C for 14 h at 34,000 rpm. Fractions were collected and assayed for radioactivity. Fractions corresponding to the 19 and 16S SV40 RNA, respectively, were separately pooled and subjected to a second cycle of sucrose gradient centrifugation. The preparations thus obtained were used for base-ratio analysis and RNase digestion.

Chemicals. Poly U, poly A, and ^{3}H-poly A were obtained from Miles Laboratories. Cytosine arabinoside-hydrochloride and pancreatic RNase A, which had been crystallized five times, were obtained from Sigma Chemical Co. RNase T1 grade B was purchased from Calbiochem. *Eschericia coli* B 4S RNA was from General Biochemicals Corp. It contained 5S RNA as an impurity. Electrophoretically purified DNase I was from Worthington Biochemical Corp.

Digestion of SV40 RNA. For T1 digestion the preparations of SV40 RNA plus added carrier tRNA were digested for 90 min at room temperature with one-tenth their weight of RNase T1 in 0.01 M Tris plus 0.001 M EDTA at pH 7.4.

SV40 RNA preparations that were tested for the presence of poly A sequences were mixed with ^{3}H-poly A and incubated in 1 ml of SDS buffer with an excess of poly U (2 μg) for 30 min at room temperature. Subsequently, 100 μg of poly A was added, and the RNA was precipitated with two volumes of ethanol. The RNA was then redissolved in 70 μliters of TMK buffer (0.01 M Tris, 0.3 M KCl, and 0.02 M $MgCl_2$) containing 10 U of RNase T1, 0.6 μg of RNase A, and 10 μg of DNase I and incubated at 37 C for 30 min. TMK buffer was added to increase the volume to 1 ml, and EDTA was added to a final concentration of 0.1 M. Oligonucleotides were precipitated with ethanol. The pellet was dissolved in H_2O and heated for 5 min at 70 C to melt double-stranded regions. After rapid cooling in ice, the oligonucleotides were precipitated with ethanol. The pellet was then again subjected to digestion with RNase T1 and A for 30 min at 37 C as described above. Finally, the oligonucleotides were reprecipitated with ethanol and dissolved in 8 M urea plus 0.1% SDS. This material was then subjected to electrophoresis on diacrylate cross-linked 10% polyacrylamide gels.

For alkaline digestion, the preparations were incubated overnight at 37 C in 0.3 M potassium hydroxide.

Electrophoretic analysis of the digestion products. The products of RNase T1 digestion were subjected to two-dimensional electrophoresis (11). The first electrophoresis was done on cellulose acetate strips (type Selectron, Schleicher & Schuell Co.) at pH 3.5, the second was done on DEAE paper (type DE 81, Whatman) at pH 1.9. The products of alkaline digestion were subjected to electrophoresis on Whatman no. 1 paper (12).

After electrophoresis, the dried papers were subjected to autoradiography to visualize the oligonucleotide-containing regions of the electropherograms. These regions could then be cut out and counted in a liquid scintillation counter.

Gel electrophoresis was done on 10% polyacrylamide gels 5% cross-linked with diacrylate (3). The buffer in which the gels were polymerized was also used for electrophoresis and contained 0.04 M Tris, 0.02 M sodium acetate, 0.001 M EDTA, 4 M urea, and 0.1% SDS at a final pH of 7.2. The gels were run at 10 V/cm. Bromophenol blue was added as a visual marker. After electrophoresis the gels were cut in 2-mm slices. If both ^{3}H and ^{32}P were to be counted, the gels were dissolved in 0.3 N NH_4OH and counted with Triton X100-toluene-2,5-diphenyloxazole-1,4-bis-2-(5-phenyloxazolyl)-benzene. Gel slices from which ^{32}P-oligonucleotides had to be eluted were counted for Cerenkov radiation at an efficiency of approximately 50% in a scintillation counter. The gels containing 4 and 5S markers were stained with methylene blue (7). Oligonucleotides were eluted from gel slices by shaking overnight in SDS buffer at 37 C.

RESULTS

In Table 1 and Fig. 1, the purification of a preparation of 19 and 16S SV40 RNA is shown. Starting with the total RNA isolated from a cytoplasmic extract of infected cells, the virus-specific RNA was isolated by hybridization to viral DNA. The viral RNA was eluted from the DNA with 90% formamide, which melts the DNA-RNA hybrids. The results of this procedure are shown in Table 1. From the total SV40-specific RNA thus isolated, the RNA species sedimenting at 19 and 16S, respectively, were purified by two cycles of sucrose gradient centrifugation (Fig. 1).

The purified 19 and 16S SV40 RNA were subjected to alkaline digestion, and the degradation products were separated by electrophoresis to determine the base composition.

In Table 2 the results of the analysis of the base composition of a number of RNA preparations are summarized. The 16S RNA appears to be very rich in adenylic acid and has a reduced content of cytidylic acid as compared to the 19S RNA. The base composition of 19S SV40 RNA, synthesized in the presence of cytosine arabinoside, a drug inhibiting the expression of late viral functions (11), was also determined. It was found that the early and late 19S RNAs have a very similar base composition, suggesting that the two species are identical.

The purified 19 and 16S RNAs were also subjected to extensive digestion with ribonuclease T1. This treatment leads to the production of numerous oligonucleotides which are separated by two-dimensional electrophoresis. The electropherograms were then subjected to autoradiography. A close visual inspection of the autoradiograms of digests of the two RNAs (Fig. 2 and 3) reveals differences in the relative darkness of spots. To obtain more quantitative information, a number of well isolated spots (Fig. 4) were cut out and counted. The spots were identified by comparing their relative

TABLE 1. *Purification of SV40-specific RNA from cytoplasmic RNA of infected BSC-1 cells*[a]

Purification stage of RNA	Total counts/ min recovered
Total cytoplasmic	$1,200 \times 10^6$
Hybridized to 50 μg of SV40 DNA on filter	5×10^6
Eluted from SV40 DNA on filter	4.8×10^6
Hybridized to blank filter	3.3×10^4
Eluted from blank filter	1.0×10^4

[a] Purification by hybridization of SV40-specific RNA starting from the cytoplasmic RNA from 20 plates of infected BS-C-1 cells, labeled with ^{32}P-orthophosphate from 52 to 90 h postinfection. The RNA hybridized to SV40 DNA was eluted with 90% formamide.

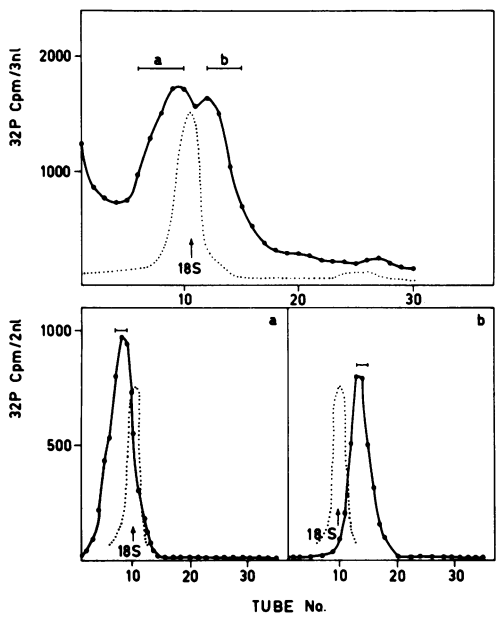

FIG. 1. *Upper panel: The SV40 RNA eluted from the SV40 DNA filter (see Table 1) was sedimented in a sucrose gradient and fractionated SV40 RNA (●——●); marker ^{32}P ribosomal RNA (. . . .) sedimented in a separate tube. SV40 RNA fractions 6 to 10 (= a) and 12 to 15 (= b) were separately pooled. Lower panel: (a) The pooled fractions (a) after resedimentation in a sucrose gradient (●——●); SV40 (. . . .) ^{32}P ribosomal RNA sedimented in a separate tube. Fractions 7 to 9 were pooled and considered as purified 19S SV40 RNA. (b) The pooled fractions after resedimentation in a sucrose gradient (●——●); SV40 RNA (. . . .) ^{32}P ribosomal RNA sedimented in a separate tube. Fractions 13 to 15 were pooled and considered as purified 16S SV40 RNA.*

positions in the fingerprints with those reported in reference 11. For each electropherogram the radioactivity found in the oligonucleotides was expressed as a percentage of the radioactivity found in guanylate plus cyclic guanylate (G + G; Fig. 4). The resulting percentages found for an oligonucleotide in different electropherograms could then be directly compared. From the results presented in Table 3 it is clear that a number of oligonucleotides were present in different relative amounts in the digests of 19 and 16S SV40 RNA. We conclude that that there is a clear difference in the base sequence of these two SV40-specific RNAs.

To estimate whether poly A is attached to these viral messengers, the following procedure was adopted: (i) ^{3}H-poly A was added to the ^{32}P-labeled viral RNAs to serve as an internal control. Then the mixture of viral RNA and ^{3}H-poly A was incubated with poly U to convert poly A to a double-stranded RNase-resistant structure, since the batches of ribonuclease T1 and A that were used contained slight traces of poly A degrading enzymes. Subsequently, unlabeled poly A was added to divert these traces of poly A degrading activities, and ribonuclease T1 plus A were added. The complete procedure for enzymatic digestion as given in Materials and Methods will select for oligonucleotide sequences resistant to RNase T1 and A. Due to the specificities of these enzymes the resulting oligonucleotides should have a high adenylic acid content.

The radioactive material precipitated by ethanol after the second enzymatic digestion was divided into two parts which were separately analyzed for size on polyacrylamide gels. One gel was dissolved and counted for ^{3}H and ^{32}P. In the experiments reported, the ^{3}H-poly A added as an internal control for poly-A degradation was in no case found to be degraded (data not shown). The second gel was counted for ^{32}P only and was then used to elute the radioactive RNA fragments which were analyzed for their base composition.

TABLE 2. *Base composition of 19 and 16S SV40 RNA*[a]

SV40 RNA species	Total radioactivity in (%)[b]			
	C	A	G	U
19S	23.0 ± 0.9	28.3 ± 0.7	23.9 ± 0.8	24.8 ± 0.7
16S	19.3 ± 0.6	34.0 ± 0.6	22.0 ± 0.2	24.7 ± 0.4
19S (Ara C)	23.5 ± 0.1	27.9 ± 0.6	24.3 ± 0.6	24.3 ± 0.4

[a] Three different preparations of 19 and 16S SV40 RNA, labeled with ^{32}P-orthophosphate from 51 to 78 h, 48 to 64 h, and 52 to 90 h postinfection, respectively, were analyzed for base composition. The first two preparations were analyzed in duplicate; the last was analyzed in triplicate. 19S (Ara C) SV40 RNA was isolated from cells infected in the presence of 5 μg of cytosine arabinoside per ml labeled with ^{32}P from 10- to 30-h postinfection. The base analysis was done in triplicate.

[b] Total radioactivity present indicated nucleotide ± the standard deviation. Abbreviations: C, cytidylic acid; A, adenylic acid; G, guanylic acid; U, uridylic acid.

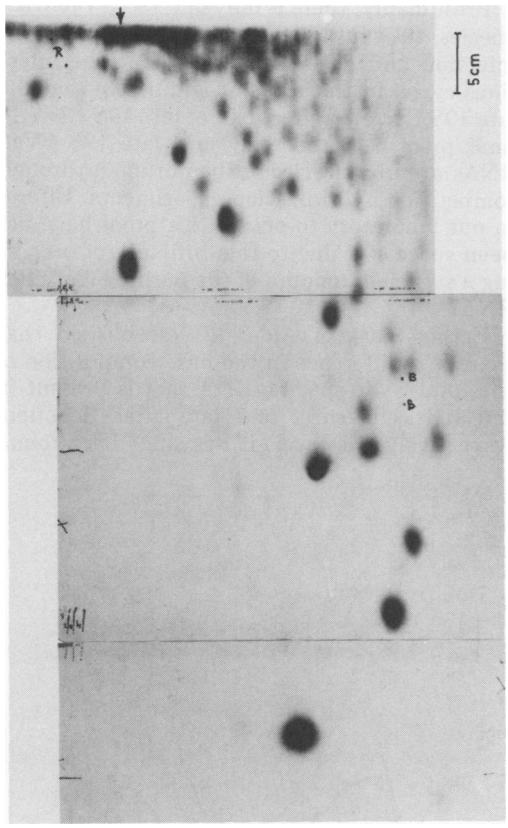

FIG. 2. *SV40 RNA (19S) labeled with ^{32}P-orthophosphate from 52 to 90 h postinfection was subjected to RNase T1 digestion and two dimensional electrophoresis. The arrow marks the region where ^{3}H-poly A was found in a control experiment. B = Blue marker (xylene cyanol FF). R = Red marker (acid fuchsine).*

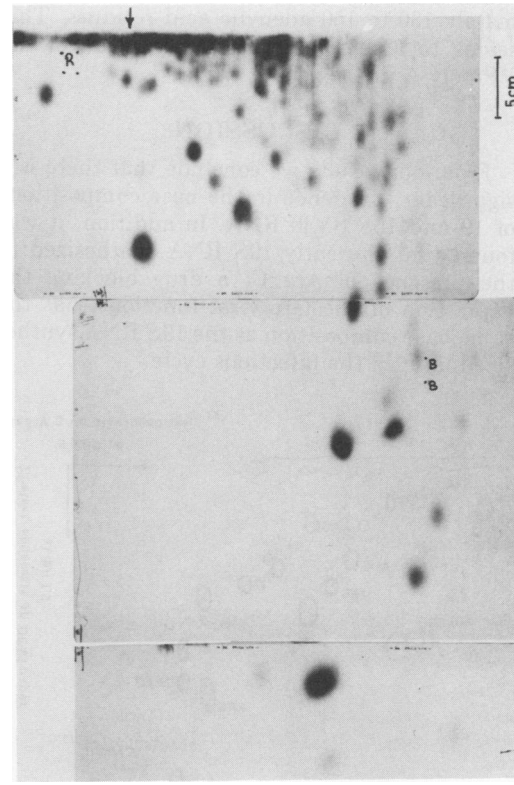

FIG. 3. *SV40 RNA (16S) labeled with ^{32}P-orthophosphate from 52 to 90 h postinfection was subjected to RNase T1 digestion and two dimensional electrophoresis. The arrow marks the region where ^{3}H-poly A was found in a control experiment. B = Blue marker (xylene cyanol FF). R = Red marker (acid fuchsine).*

From Fig. 5 it can be seen that the ^{32}P radioactivity is present in two peaks, one having the mobility of low molecular weight products, and a second peak migrating more slowly than 5*S* RNA. The radioactive material was eluted and analyzed. The base composition of the pooled fractions migrating in the gels between 4.5 and 6.5 cm was (mol%) cytidylic acid (C), 20.4; adenylic acid (A), 37; guanylic acid (G), 27.6; and uridylic acid (U), 15.0 mol% for the oligonucleotide fragments from 19*S* SV40 RNA and C, 19.4; A, 19.0; G, 34.1; and U, 27.5 for the oligonucleotides from the 16*S* SV40 RNA and showed no clear enrichment for adenylic acid residues. This, however, was the case for the more slowly migrating oligonucleotides. The oligonucleotides migrating more slowly than the 5*S* RNA marker (slower than about 2.7 cm) were pooled, and the base composition was found to be (mol%) C, 3.3; A, 96.2; G, 0.3; and U, 0.2, for fragments from the 19S SV40 RNA and C, 2.4; A, 95.2; G, 1.8; and U, 0.6 for the fragments from 16*S* SV40 RNA. This fraction was, therefore, considered to be poly A. The significance of the presence of small amounts of the remaining three nucleotides was not investigated.

An estimate of the size of the poly A sequence in these SV40 RNAs was made on the basis of the initial amounts of ^{32}P-SV40 RNA subjected to enzymatic breakdown, the amount of ^{32}P-oligonucleotides (= poly A) migrating more slowly than 5*S* after the enzymatic degradation, and the percentage of ^{3}H-poly A recovered after the enzymatic digestions. The 19*S* SV40 RNA contained about 4.2% of its radioactivity in poly A, and the 16*S* SV40 RNA contained about 7.5%. If the 19 and 16*S* RNAs contain 3,000 and 2,000 nucleotides, respectively (15), these SV40 RNAs would have a poly A sequence of approxi-

mately 130 to 150 adenylic acid residues. This seems to agree well with the position of the ^{32}P-poly A in the gels.

DISCUSSION

From our results we conclude that there is a significant difference in the base compositions of 19 and 16S SV40 RNA. In addition, it was found that the early 19S RNA synthesized in the presence of Ara C, a drug blocking the expression of the late viral functions, has the same base composition as the 19S RNA synthesized later in the infectious cycle.

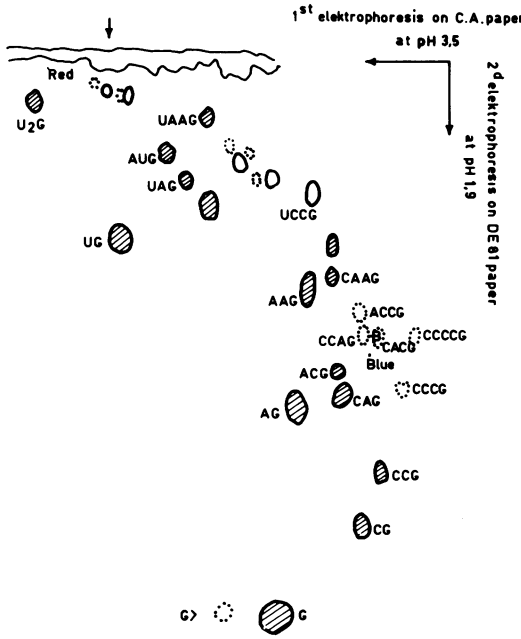

FIG. 4. *This diagram shows the relative position of some of the oligonucleotides appearing in the electropherograms of RNase T1 digests of SV40 RNA and indicates the base sequence of a number of these oligonucleotides. Oligonucleotides were identified by comparison with data presented in reference 11.*

Presumably, there is only one 19S SV40 RNA species, the synthesis of which starts early after infection and continues throughout the infectious cycle. In that case, it is unlikely that the late 19S RNA is a precursor of late 16S RNA. A final proof that the early and late 19S SV40 RNAs are identical could be obtained through competition hybridization experiments. Efforts in our laboratory to obtain this proof have not been successful due to the difficulty of preparing a sufficient amount of the purified early 19S RNA.

Earlier work of Aloni (2) established that there is a difference in the base composition of the total SV40-specific RNA that is present in infected cells early and late after infection, respectively. However, it has since been found

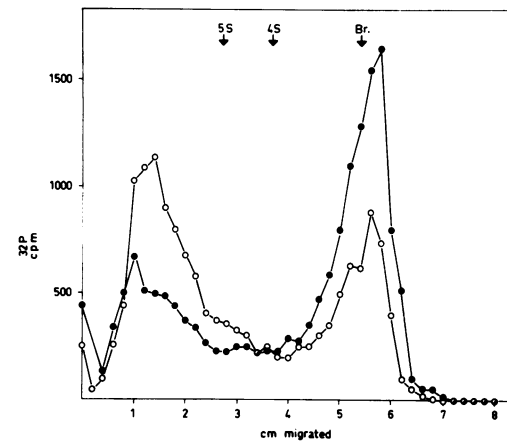

FIG. 5. *Oligonucleotides precipitated by ethanol after digesting SV40 RNA with ribonucleases T1 plus A were electrophoresed on polyacrylamide gels. The SV40 RNA was isolated from cells labeled with ^{32}P from 41 to 56 h after infection with SV40 virus. Symbols: (●——●) Oligonucleotides from 19S SV40 RNA, (○——○) oligonucleotides from 16S SV40 RNA. The positions of bromophenol blue in the gels and the 4 and 5S markers in a control gel are indicated by arrows.*

TABLE 3. *Distribution of radioactivity in oligonucleotides produced by digestion of 19 and 16S SV40 RNA with RNase T1*[a]

SV40 RNA species	Oligonucleotides present								
	CG	C2G	AG	CAG	ACG	C3G	C2AG	CACG	C4G
19S	43.8 ± 0.4	21.7 ± 0.4	44.3 ± 0.9	17.7 ± 0.3	8.1 ± 0.1	6.9 ± 0.1	4.0 ± 0.1	4.1 ± 0.1	3.6 ± 0.1
16S	17.7 ± 1.5	9.0 ± 1.0	57.0 ± 3.4	24.7 ± 0.1	5.2 ± 0.2	2.9 ± 0.0	4.3 ± 0.5	2.5 ± 0.3	1.4 ± 0.1
	AC2G	A2G	CA2G	UG	AUG	UAG	UC2G	U2G	UA2G
19S	3.7 ± 0.3	17.7 ± 1.3	7.3 ± 0.1	40.5 ± 1.5	18.4 ± 0.7	8.7 ± 0.1	5.6 ± 0.5	13.8 ± 0.7	9.0 ± 0.4
16S	2.8 ± 0.3	29.5 ± 1.8	6.6 ± 0.5	63.1 ± 4.4	27.2 ± 2.0	10.8 ± 0.6	12.6 ± 0.7	18.0 ± 2.3	8.7 ± 0.6

[a] SV40 RNA (19 and 16S) labeled with ^{32}P-orthophosphate from 52 to 90 h postinfection were subjected to RNase T1 digestion. The oligonucleotides were separated by electrophoresis and counted. The numbers in the table indicate the amount of radioactivity in each oligonucleotide, expressed as a percentage of the counts found in guanylate plus cyclic guanylate in the corresponding electropherogram. The results presented are the average of two digestion experiments ± the standard deviation.

that the total SV40 RNA is made up of a number of distinct fractions, i.e., the 19S present early and late after infection, the 16S present late after infection only (15), and the large heterogeneous RNA (13). This large heterogeneous RNA has since been found to contain host-cell specific sequences in covalent linkage to SV40-specific sequences. In addition, total SV40 RNA has been found to contain self-complementary sequences (1, 6, 9). It is, therefore, not possible to directly compare the results in reference 1 with the results of the present study.

As expected from the differences in the base composition, the ribonuclease T1 fingerprints indicate that there is a difference in the base sequence of the 19 and 16S SV40 RNA present late after infection.

The results of our study on the presence of poly-A sequences in 19 and 16S SV40 RNA are confirming a previous report (14) and show that 19 and 16S RNA contain a poly-A sequence of about 130 to 150 adenylic acid residues.

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