JOURNAL OF VIROLOGY, Aug. 1974, p. 249-260
Vol. 14, No. 2

Characterization of Late Polyoma mRNA

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Received for publication 15 March 1974

Polyoma-infected mouse kidney cell cultures were labeled with [^{3}H]uridine for 3 h late in the lytic cycle (26 to 29 h after infection) and RNA was extracted from cytoplasm and nuclei and from isolated polyribosomes. Sedimentation velocity analysis in sucrose gradients showed that polyoma-specific "giant" and 26*S* RNAs are present exclusively in the nucleus. RNA associated with cytoplasmic polyribosomes was analyzed by sedimentation in aqueous sucrose density gradients and dimethylsulfoxide sucrose gradients, as well as by polyacrylamide gel electrophoresis. Polyoma-specific RNA in polyribosomes consists of at least two classes, with sedimentation coefficients of 16 (major fraction) and 19*S* (minor fraction) in aqueous sucrose gradients and 15 and 17*S*, respectively, in dimethylsulfoxide gradients. Estimates based on dimethylsulfoxide gradient and analysis suggest a molecular weight of approximately 500,000 for 16*S* RNA and 700,000 for 19*S* RNA. These polyoma RNAs seem to undergo reversible conformational changes under the different conditions of analysis. We cannot exclude the possibility that they contain more than one molecular species.

Earlier studies showed that RNA extracted from primary mouse kidney cell cultures infected with polyoma virus and labeled late in the infectious cycle with [^{3}H]uridine contains three size classes of polyoma-specific RNA (1): (i) heterogeneous "giant" molecules with sedimentation coefficients between approximately 30 and 80*S*; (ii) RNA molecules with a sedimentation coefficient of about 26*S*, which may have the size of the transcript of one strand of polyoma deoxyribonucleic acid (DNA); (iii) a class of at least two RNA species with sedimentation coefficients between 16 and 20*S*.

The large RNAs are the predominant species labeled during a brief (10- to 30-min) exposure of the cultures to [^{3}H]uridine; sedimentation analysis in dimethylsulfoxide (DMSO) gradients showed that they are not aggregates of smaller molecules (1). In contrast, the 26 and 16 to 20*S* species become apparent in RNA labeled for longer periods (1 h or more).

In the present work, we first attempted to locate these RNA classes in the cell and to determine which of them may correspond to polyoma-specific mRNA. We therefore labeled mouse kidney cell cultures with [^{3}H]uridine for 3 h late (26 to 29 h) during the lytic infection. At this time the rate of synthesis of polyoma RNA is approaching the maximum. From the separated nuclei, cytoplasm, and polyribosomes, we extracted total RNA and determined the properties of polyoma-specific RNA.

The results reported in this paper show that polyoma-specific RNA sedimenting with coefficients of 26*S* or higher is found only in the nucleus, whereas the 16 to 20*S* viral RNAs are found mainly in the cytoplasm, in association with the polyribosomes. Detailed analysis of the polyribosome-associated, hybridizable polyoma-specific RNA reveals the presence of at least two distinct classes, which we designate operationally as late 19 and 16*S* mRNAs.

MATERIALS AND METHODS

Cell fractionation and isolation of polyribosomes. Primary mouse kidney cell cultures (28) in 88-mm petri dishes were infected (18) with wild-type polyoma virus 1 day after confluence. Cells were usually labeled for about 3 h at 37 C with 200 to 300 μCi per dish of [5-^{3}H]uridine (New England Nuclear Corp., about 25 Ci/mmol) in 1 ml of warm reinforced Eagle medium. At the end of the labeling period, the radioactive medium was replaced by medium containing 50 μg of emetine hydrochloride per ml (Fluka) and the cultures were incubated for a further 5 min at 37 C (4). The cultures were then put on ice, washed quickly with ice-cold isotonic buffer (0.25 M sucrose, 10 mM triethanolamine, pH 7.6, 25 mM NaCl, 5 mM $MgCl_2$) and lysed (2) by the addition of a 1% solution of Nonidet-P40 (BDH Chemicals, Ltd.) in the same buffer (1 ml for about 5×10^7 to 8×10^7 cells). The lysate was collected by gentle scraping and careful pipetting to disrupt aggregates. Nuclei and large particles were sedimented by centrifugation at 12,000

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× g for 20 min, the supernatant being the "cytoplasmic extract." For isolation of polyribosomes, the cytoplasmic extract was mixed with 1/10 volume of 10% sodium deoxycholate to release membrane-bound polyribosomes, layered onto a 15 to 50% (wt/vol) sucrose gradient in 50 mM triethanolamine (pH 7.6), 25 mM NaCl, 1 mM $MgCl_2$, and centrifuged at 40,000 rpm (200,000 × g_{av}) for 75 min at 2 C in a Spinco SW 40 rotor. Gradients were collected through a continuous-flow ultraviolet absorbancy monitor (Zeiss) to analyze the optical density profile. For determination of radioactivity, samples of each fraction were dried on filter-paper disks, which were washed at 4 C in 5% trichloroacetic acid, dried, and counted in toluene-based scintillation fluid.

Extraction of RNA. (i) Sodium dodecyl sulfate (SDS) was added to a final concentration of 1% to cytoplasmic extracts (diluted 1:5 with 0.01 M sodium acetate, pH 5.1) or to pooled polyribosome fractions from sucrose gradients. RNA was immediately extracted at room temperature (20 to 22 C) with an equal volume of a mixture of phenol-chloroform-isoamylalcohol in the ratio 50:50:1 (16) in the presence of 2 mM EDTA. (ii) Total RNA was extracted as previously described (1, 20) with hot (65 C) phenol-1% SDS in 0.01 M sodium acetate (pH 5.1).

All RNA preparations were precipitated twice with ethanol and incubated at 0 C for 30 min with 10 μg of deoxyribonuclease I per ml (ribonuclease-free, Worthington) in 0.01 M sodium acetate (pH 5.1), 2 mM $MnCl_2$ to eliminate possible contamination with viral DNA (1). The enzyme was removed either by adsorption to Macaloid (100 μg/ml) and subsequent centrifugation, or by two extractions with phenol-chloroform-isoamylalcohol as above. The final RNA preparations were then reprecipitated with ethanol.

DNA-RNA hybridization. DNA-RNA hybridization was performed in 100 μliters of 4× SSC (SSC: 0.15 M NaCl, 0.015 M Na citrate, pH 7.4) at 65 C for 40 to 48 h with two 3.5-mm membrane filters, each containing 0.2 μg of denatured, purified polyoma DNA I ("53S"). The filters were subsequently washed and incubated with 5 μg of RNase per ml in 4× SSC for 1 h at room temperature, washed extensively, dried, and their radioactivity measured. Polyoma RNA is defined as the radioactivity remaining bound to RNase-treated filters. Blank filters incubated together with DNA-containing filters retained less than 0.01% of the radioactivity present in the hybridization mixture. Details of the hybridization technique are contained in reference 1.

Sedimentation in sucrose gradients. RNA samples were analyzed in 4.4-ml, 15 to 30% (wt/wt) sucrose gradients made in 10 mM triethanolamine (pH 7.4), 50 mM NaCl, 1 mM EDTA, and centrifuged in a Spinco SW 56 rotor at 54,000 rpm (300,000 × g_{av}) and 20 C. Fractions were collected by puncturing the bottom of the tube and samples were dried on filter paper disks, which were washed with cold 5% trichloroacetic acid and counted. The remainder of each fraction was adjusted to 4× SSC by adding 20× SSC and was hybridized.

Sedimentation in DMSO. Sedimentation in DMSO was performed as described (1).

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was performed in a buffer containing 40 mM triethanolamine (pH 7.4), 20 mM sodium acetate, 2 mM EDTA, 2.5% glycerol, 0.2% SDS (12, 1). Gels (10 cm) containing 3% acrylamide and 0.15% bis-methylene acrylamide were polymerized in 0.6-cm (inside diameter) quartz tubes above 1.5-cm cushions of 5% polyacrylamide. Electrophoresis was at 10 V/cm and 22 C. The frozen gels were sliced into 2-mm disks. The RNA in each slice was eluted with 100 μliters of 4× SSC at 65 C overnight. A sample of the eluate was dried on filter-paper disks, which were washed with cold 5% trichloroacetic acid and counted. The remainder of the eluate, still containing the gel slice, was subsequently hybridized with polyoma DNA.

RESULTS

Sedimentation velocity analysis of polyoma RNA extracted from cytoplasm or nuclei of infected cells. Polyoma-infected mouse kidney cell cultures were labeled with [^{3}H]uridine for 3 h, between 26 and 29 h after infection. At this time, virtually all cells contain intranuclear polyoma-specific T-antigen and 70 to 80% of the cells synthesize cell and viral DNA, as determined by autoradiography (25). The cultures were lysed with the nonionic detergent NP-40 (2), applied directly onto the cell monolayer; nuclei and cytoplasm were then separated by centrifugation, as described in Materials and Methods.

Figure 1 shows the sedimentation pattern of RNA extracted with phenol-chloroform at room temperature from the cytoplasm. Total radioactivity is present in 28 and 18S ribosomal RNA and 4 to 5S RNA, whereas no radioactivity is associated with RNA sedimenting faster than 28S. The bands corresponding to 28 and 18S ribosomal RNAs contain approximately equal amounts of radioactivity; this pattern is different from that of RNA extracted from total cells, where the 28S RNA band always contains more radioactivity than the 18S band (see ^{14}C-marker RNA). It probably reflects the more rapid transport of 18S ribosomal RNA into the cytoplasm (24). It should also be noted that the band containing 28S ribosomal RNA extracted at room temperature sediments slightly faster than the corresponding band of ^{14}C-marker RNA, extracted by the hot-phenol procedure (65 C). The increased sedimentation velocity is probably due to the presence of hydrogen-bonded "7S RNA" (13), which is dissociated under denaturing conditions, such as elevated temperatures or concentrated DMSO.

Polyoma RNA was determined in each fraction by hybridization to polyoma DNA immobi-

lized on membrane filters (1) and present in excess, as determined in previous DNA-saturation hybridization experiments with total infected cell RNA (unpublished data). The bulk of polyoma RNA formed a rather broad band with a sedimentation coefficient of about 16S and a slightly faster sedimenting shoulder. We will show in the subsequent sections that this band comprises two classes of polyribosome-associated RNA, designated as polyoma-specific "16S" and "late 19S" RNAs. They correspond to the 16 to 20S RNA found earlier in total cell extracts (1). Only very small fractions of polyoma RNA were found to sediment slower than 16S and faster than 19S, whereas none was found to sediment faster than 24S. Thus, polyoma RNAs with sedimentation coefficients of 26S or higher, which are found in total cell extracts, are absent in RNA extracted from the cytoplasm.

These large viral RNAs were found in the nuclear pellet remaining after separation of the cytoplasmic fraction. RNA was extracted from such a pellet by the hot-phenol procedure (20) and analyzed by sedimentation in linear sucrose density gradients (Fig. 2). The results show that total radioactivity is present in large, heterogeneous RNA, ribosomal precursor RNAs, ribosomal 28S RNA, and, to a lesser extent, 18 and 4 to 7S RNAs. The presence of 18S ribosomal RNA probably reflects a contamination of the nuclear preparation with cytoplasm. The bulk of polyoma RNA consists of heterogeneous molecules with sedimentation coefficients larger than 26S and a peak of 26S RNA, whereas only a small fraction of the total viral RNA exhibits sedimentation coefficients of 20S or less.

Preparation of polyribosomes. Cytoplasmic extracts were prepared as described above, both from infected and from uninfected cultures. To prevent ribosome run-off during manipulations, emetine was added to the culture medium before extraction (4). The cytoplasmic extracts were treated with deoxycholate to release membrane-bound polyribosomes. The polyribosomes were then separated from soluble components, single ribosomes, and ribosomal subunits by sedimentation in sucrose gradients. Figure 3 shows the optical density profile of polyribosomes isolated from cultures 30 h after infection. This profile is indistinguishable from that observed with polyribosomes prepared from mock-infected parallel cultures.

For RNA analysis, polyribosomes were pre-

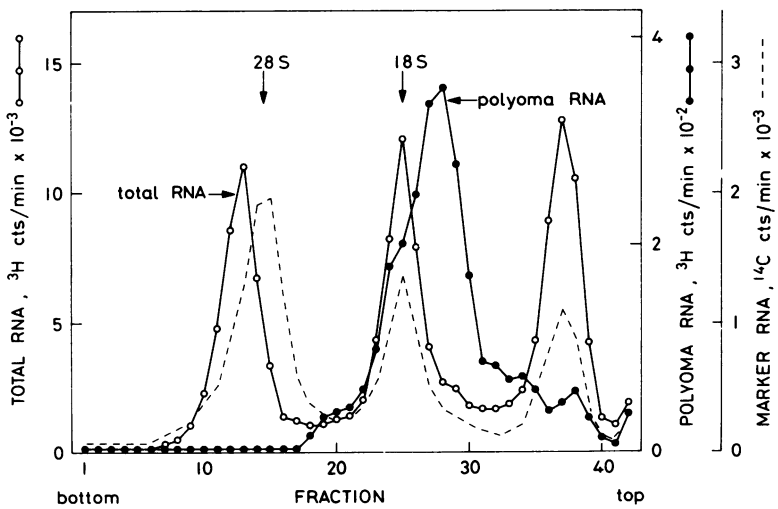

FIG. 1. *Sucrose gradient sedimentation analysis of cytoplasmic RNA from polyoma-infected mouse kidney cells labeled with [³H]uridine (200 μCi/ml) between 26 and 29 h after infection. The cytoplasmic extract (prepared as described in Materials and Methods) was diluted fivefold with 0.01 M sodium acetate (pH 5.1) containing SDS to a final concentration of 1%, and RNA was extracted with phenol-chloroform-SDS at room temperature (20 to 22 C). About 13 μg of [³H]RNA (20,000 counts per min per μg) was mixed with about 0.7 μg of mouse cell RNA labeled with [¹⁴C]uridine for 24 h (40,000 counts per min per μg) and extracted with phenol-SDS at 65 C. Centrifugation was through a 15 to 30% sucrose gradient in a Spinco SW 56 rotor (54,000 rpm, 3 h, 20 C). One-third of each fraction was counted, and two-thirds were hybridized to polyoma DNA. "Total RNA" represents the total ³H counts per minute available for hybridization in each fraction; "polyoma RNA" represents the ³H counts per minute hybridized per fraction. All values are corrected for background and counting overlap. Blank filters contained less than 2 counts/min.*

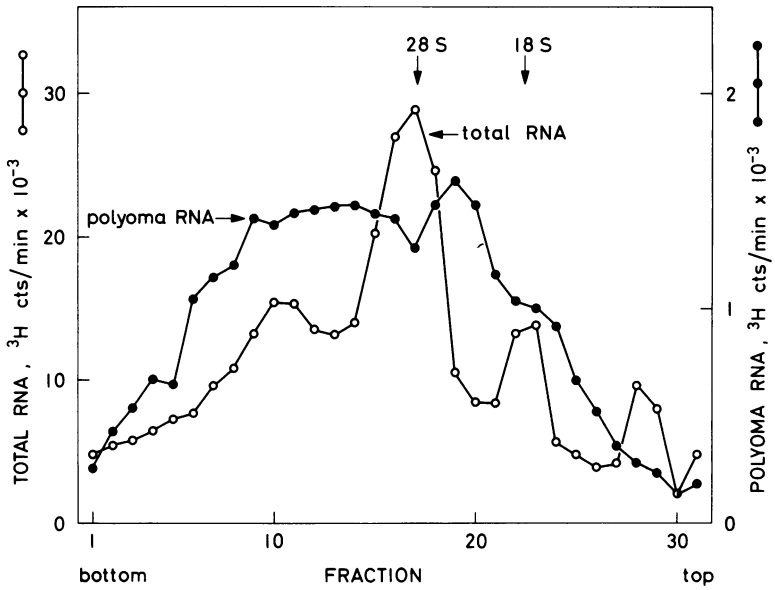

FIG. 2. *Sucrose gradient sedimentation analysis of RNA from the nuclear fraction of polyoma-infected mouse kidney cells labeled with [^{3}H]uridine (250 μCi/ml) between 26 and 29 h after infection. The nuclear pellet was washed once with cold phosphate-buffered saline before RNA extraction with phenol-SDS at 65 C. About 14 μg of [^{3}H]RNA (33,000 counts per min per μg) was centrifuged on a 4.4-ml 15 to 30% (wt/wt) sucrose gradient in a Spinco SW 56 rotor at 54,000 rpm (300,000 × g_{av}) for 105 min at 20 C, with ^{14}C-labeled mouse cell RNA marker in the same tube. One-fourth of each fraction was counted (values are corrected for background and counting overlap). "Total RNA" represents the calculated total ^{3}H radioactivity in the remainder of each fraction, which was hybridized to polyoma DNA. "Polyoma RNA" represents the ^{3}H counts per minute hybridized per fraction. Sedimentation is from right to left. The arrows mark the positions of the ^{14}C-labeled ribosomal RNAs.*

pared from infected cultures labeled with [^{3}H]uridine for 3 h (26 to 29 h after infection). Gradient fractions containing polyribosomes (see Fig. 3) were pooled; RNA was extracted at room temperature (20 to 22 C) with phenol-chloroform-SDS and then hybridized with polyoma DNA (see Materials and Methods). Under the hybridization conditions used, 70 to 80% of the radioactive, virus-specific RNA was removed from the hybridization mixture during the first cycle of hybridization. Polyoma RNA accounted for 1 to 3% of total radioactivity, as determined in 11 experiments.

Sedimentation pattern of polyribosomal RNA in sucrose density gradients. In seven independent experiments, mouse kidney cell cultures were labeled with [^{3}H]uridine from 26 to 29 h after infection and polyribosomes were prepared as described in the text to Fig. 3. RNA was extracted with phenol-chloroform-SDS and then sedimented through linear sucrose density gradients. A representative result is shown in Fig. 4. The patterns of ribosomal RNA and of polyoma RNA (16 and 19S) are similar to those of RNA extracted from the cytoplasm (see Fig. 1). However, as expected, less total radioactiv-

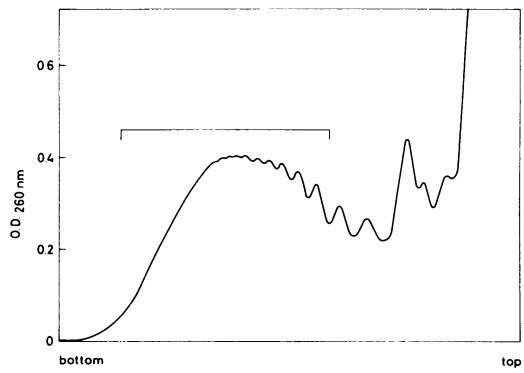

FIG. 3. *Sedimentation profile of polyribosomes from polyoma-infected mouse kidney cells 30 h after infection. Five infected cultures were lysed in 0.5 ml of 1% NP-40. A cytoplasmic extract, prepared as described in Materials and Methods, was centrifuged through a 12-ml 15 to 50% (wt/vol) sucrose gradient for 75 min at 40,000 rpm (200,000 × g_{av}), 2 C, in a Spinco SW 40 rotor. The gradient was collected through a flow cell in a Zeiss recording spectrophotometer. Sedimentation is from right to left. The bar indicates the part of the gradient which was pooled as the "polyribosome fraction" in a standard experiment.*

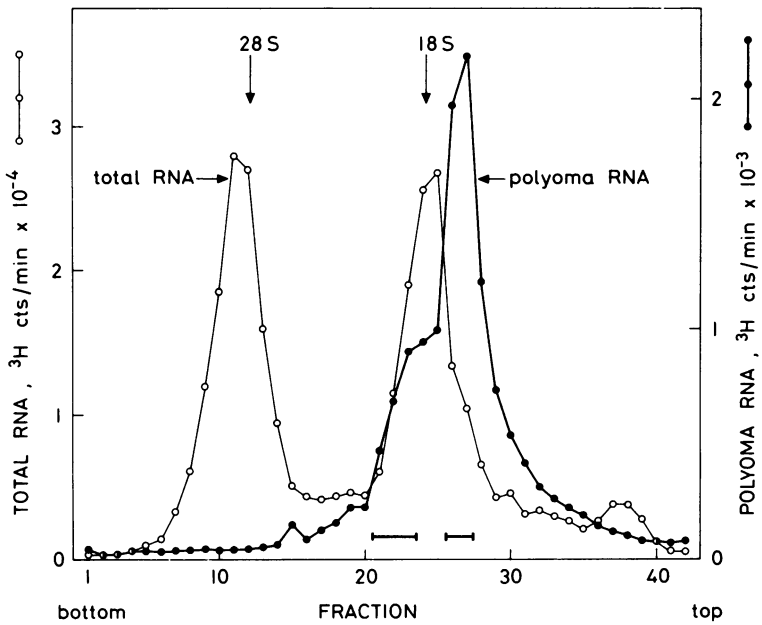

FIG. 4. *Sucrose gradient sedimentation analysis of polyribosomal RNA. Polyoma-infected mouse kidney cell cultures were labeled with [^{3}H]uridine (300 μCi/ml) between 26 and 29 h after infection, and RNA was extracted from the isolated polyribosomes with phenol-chloroform-SDS at room temperature. About 20 μg of [^{3}H]RNA (18,000 counts per min per μg) was mixed with about 1 μg (40,000 counts/min) of ^{14}C-labeled RNA from uninfected mouse kidney cells extracted with phenol-SDS at 65 C. Centrifugation was through a 15 to 30% sucrose gradient in a Spinco SW 56 rotor (54,000 rpm, 3 h, 20 C). Arrows indicate sedimentation values based on the profile of the ^{14}C-marker. "Total RNA" represents the total ^{3}H counts per minute per fraction (after correction for ^{14}C spill-over), calculated from a sample which was counted. "Polyoma RNA" represents the ^{3}H counts per minute per fraction hybridized to polyoma DNA. Two more gradients, containing about 50 μg of the [^{3}H]RNA each, were run in parallel to this; a sample of each fraction was counted, giving a profile which was similar to the one shown here. In these two gradients, the fractions corresponding to the bars (21 to 23 and 26 to 27) were separately pooled and precipitated with ethanol, after addition of 10 μg of E. coli transfer RNA, and the RNA was used for further analyses (see Fig. 7 and 9).*

ity is present in the 4 to 5*S* region. In all experiments, 16*S* virus-specific RNA was the predominant band, whereas the relative amounts of 19*S* RNA varied only slightly.

Evidence that polyoma-specific 16 and 19S RNAs are polyribosome associated. EDTA dissociates polyribosomes into mRNA (in the form of ribonucleoprotein particles; 14, 15) and ribosomal subunits, which both sediment considerably slower than polyribosomes. If polyoma-specific 16 and 19*S* RNAs are truly associated with polyribosomes, then treatment with EDTA should lead to the disappearance both of polyribosomes and of virus-specific RNA from the "polyribosome region" in sucrose gradients. The following experiment shows that this is the case.

Cultures were labeled with [^{3}H]uridine from 26.5 to 30 h after infection. The cytoplasmic extract was divided into two samples, one of which served as an untreated control; to the other sample, EDTA was added to a final concentration of 20 mM (see legend to Fig. 5). Both samples were then immediately sedimented through parallel sucrose density gradients. A sample of each fraction was counted; the superimposed radioactivity profiles obtained from the two gradients are shown in Fig. 5. The untreated control shows the expected pattern of polyribosomes, which contain about 40% of total radioactivity present in the gradient; in contrast, in the EDTA-treated sample only a very small fraction (3.5%) of total radioactivity is present in the polyribosome region, whereas the remainder is found at the top of the gradient. Fractions 4 to 22 from the polyribosome region of each gradient were separately pooled and RNA was extracted with phenol-SDS. Both preparations were then separately sedimented through sucrose density gradients. Polyoma RNA from the untreated control exhibited the pattern expected for polyoma RNA extracted from polyribosomes (see Fig. 4); in contrast, only trace amounts of viral RNA were found in

the gradient containing RNA extracted from the EDTA-treated sample.

Sedimentation pattern of polyribosomal RNA in DMSO gradients. In an attempt to eliminate the effects of molecular conformation of polyoma RNA on its sedimentation behavior, we performed sedimentation velocity analyses in DMSO gradients under denaturing conditions (22). In three independent experiments, mouse kidney cell cultures were labeled with [^{3}H]uridine for 3 h, between 26 and 29 h after infection; polyribosomes were prepared, and polyribosomal RNA was extracted with phenol-chloroform-SDS. The RNA was dissolved in 90% DMSO and then analyzed in sucrose density gradients containing 99% DMSO (1, 22); as a sedimentation marker, ^{14}C-labeled mouse cell RNA was added, as used in the experiment shown in Fig. 1. A representative pattern is shown in Fig. 6. The profile of the ^{3}H-labeled ribosomal RNA is similar to that observed in aqueous sucrose density gradients. It should be noted, however, that the ^{3}H- and ^{14}C-labeled 28*S* ribosomal RNA bands overlap. This can be explained by the removal in 99% DMSO of 7*S* RNA from the ^{3}H-labeled 28*S* ribosomal RNA (cf. Fig. 1).

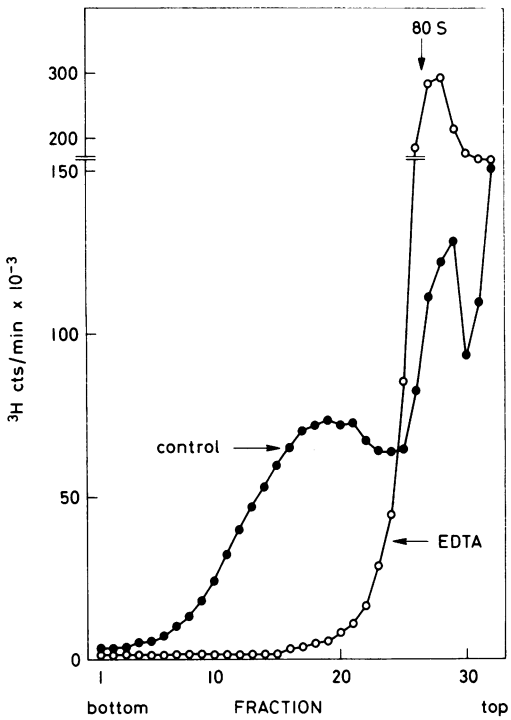

FIG. 5. *Effect of EDTA treatment on the sedimentation behavior of cytoplasmic ribosomal particles. Eight infected cultures were labeled with [^{3}H]uridine (300 μCi/ml) between 26.5 and 30 h after infection. To one-half (1.0 ml) of the cytoplasmic extract, 0.5 M EDTA (pH 7.2) was added to a final concentration of 20 mM, and one-fourth of it was immediately centrifuged through a 15 to 50% (wt/vol) sucrose gradient for polyribosome analysis (SW 40, 40,000 rpm, 75 min, 2 C) containing 1 mM EDTA ("EDTA"). One-fourth of the other, untreated half was centrifuged through a parallel gradient without EDTA ("Control"). A sample of each fraction (25%) was acid-precipitated and counted. "80S" indicates the position of single ribosomes, as determined from the optical density tracing. Two gradients containing the remainder of each sample of EDTA-treated and untreated cytoplasm were run in parallel to those shown here; fractions 4 to 22 were pooled and RNA extracted with phenol-SDS for further analyses.*

In all experiments, the profile of total radioactivity showed a small shoulder at about 15*S*. Since this shoulder was never found in the RNA from uninfected cultures, it is probably due to the presence of viral RNA. In fact, polyoma RNA sediments in DMSO gradients as a rather uniform band with a sedimentation coefficient of 15*S* with reference to ribosomal RNAs. Rather surprisingly, no evidence for the presence of a polyoma-specific 19*S* RNA could be obtained in DMSO gradients, although the resolution is comparable to that of aqueous sucrose density gradients (cf. Fig. 1 and 4). We considered the possibility that either 19*S* RNA was cleaved in DMSO, or that its sedimentation velocity relative to 16*S* RNA decreased, due to a change in conformation.

The following experiments tend to support the hypothesis that in 99% DMSO 19*S* RNA undergoes a conformational change, which is reversible after the removal of DMSO.

Polyoma-specific late 19S RNA exhibits in DMSO gradients a sedimentation coefficient of 17S. An aliquot of the [^{3}H]uridine-labeled polyribosomal RNA shown in Fig. 4 was sedimented in parallel through an aqueous sucrose density gradient. From this second gradient, the fractions containing polyoma-specific 16 and 19*S* RNAs, respectively, were pooled as indicated in Fig. 4; the two RNA samples were precipitated with ethanol in the presence of carrier *E. coli* transfer RNA, resuspended, and then separately sedimented through 99% DMSO gradients. As sedimentation marker, ^{14}C-labeled *E. coli* 16*S* ribosomal RNA was used in both gradients. The results (Fig. 7A and B) show that also under denaturing conditions two classes of polyoma RNA can be distinguished.

As shown in Fig. 7A, polyoma RNA from the 16*S* region of a sucrose gradient sedimented in DMSO slightly slower than the 16*S* marker, and formed a relatively uniform and narrow band with a sedimentation coefficient of 15*S* (relative

to the 16 and 18S RNAs). The profile of total radioactivity shows a major band around 15S, which mainly corresponds to polyoma RNA, whereas the minor band corresponds to 18S ribosomal RNA.

In contrast, polyoma RNA from the 19S region of the same sucrose gradient sedimented in DMSO slightly faster than the 16S marker, with a sedimentation coefficient of about 17S (Fig. 7B). The band containing polyoma RNA is somewhat broader than both the bands of 16S marker and of 16S polyoma RNA sedimented under the same conditions (Fig. 7A); it remains unknown whether this increased broadness is due to a molecular heterogeneity of the 19S RNA or to a contamination by 16S RNA (15S in DMSO). However, the results presented below tend to rule out a major contamination by 16S RNA.

Analysis of polyribosomal RNA by gel electrophoresis. Polyribosomal RNA extracted from mouse kidney cell cultures labeled with [^{3}H]uridine for 3 h between 26 and 29 h after infection was also analyzed by electrophoresis in 3% polyacrylamide gels. The results in Fig. 8 show that total radioactivity is mainly present in 28 and 18S ribosomal RNAs, which are resolved as two narrow bands. In contrast, polyoma RNA is more broadly distributed in a band and a shoulder between 28 and 18S ribosomal RNAs. The pattern shown in Fig. 8 is representative of those observed with RNAs obtained and analyzed in five independent experiments. We will show below how it relates to the 19 and 16S RNA classes of sucrose gradients.

Electrophoresis of separated polyoma-specific 16 and 19S RNAs in polyacrylamide gels. Another aliquot of the polyribosomal RNA shown in Fig. 4 was sedimented through a parallel sucrose gradient; from this third gradient, the fractions corresponding to the 16 and 19S regions were again pooled (see Fig. 4). The two RNA samples were precipitated with ethanol in the presence of carrier *E. coli* transfer RNA, resuspended, and analyzed by electrophoresis in polyacrylamide gels under the same conditions as in Fig. 8.

Figure 9A shows that polyoma RNA from the 16S region of a sucrose gradient forms in polyacrylamide gels a relatively broad band which migrates slightly slower than 18S ribosomal RNA. Figure 9B shows that polyoma RNA from

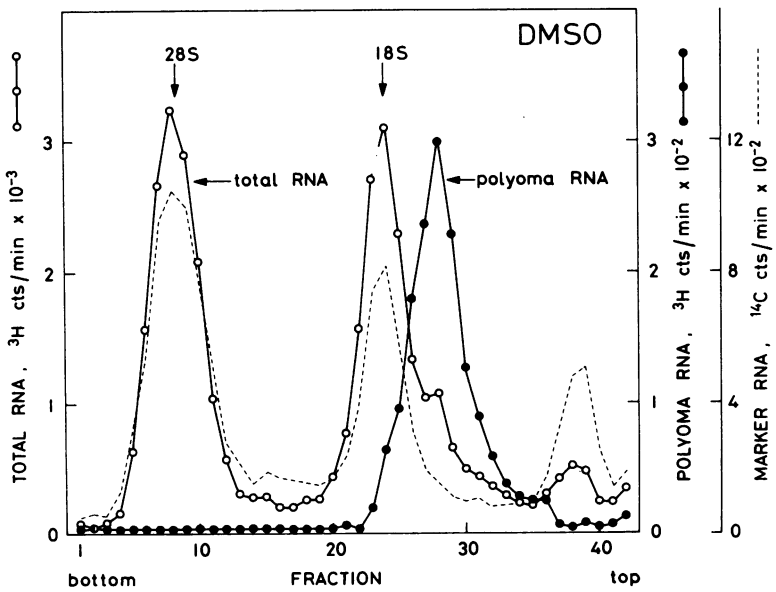

FIG. 6. *DMSO-sucrose gradient of polyribosomal RNA. Polyoma-infected mouse kidney cell cultures were labeled with [^{3}H]uridine between 26 and 29 h after infection and RNA was extracted from the isolated polyribosomes with phenol-chloroform-SDS at room temperature. About 13 μg of [^{3}H]RNA (9,300 counts per min per μg) was mixed with about 0.7 μg of ^{14}C-labeled mouse kidney cell RNA (40,000 counts per min per μg) and centrifuged through a gradient made in 99% DMSO, 1 mM EDTA, containing 0 to 10% sucrose and 10 to 89% deuterated DMSO (Spinco SW 56 rotor, 48,000 rpm, 4.5 h, 27 C). A sample of each fraction was counted ("total RNA" = total ^{3}H counts per minute per fraction, corrected for ^{14}C spill-over); the remainder was precipitated with ethanol in the presence of carrier E. coli transfer RNA, resuspended, and hybridized to polyoma DNA ("polyoma RNA" = ^{3}H counts per minute per fraction hybridized to polyoma DNA).*

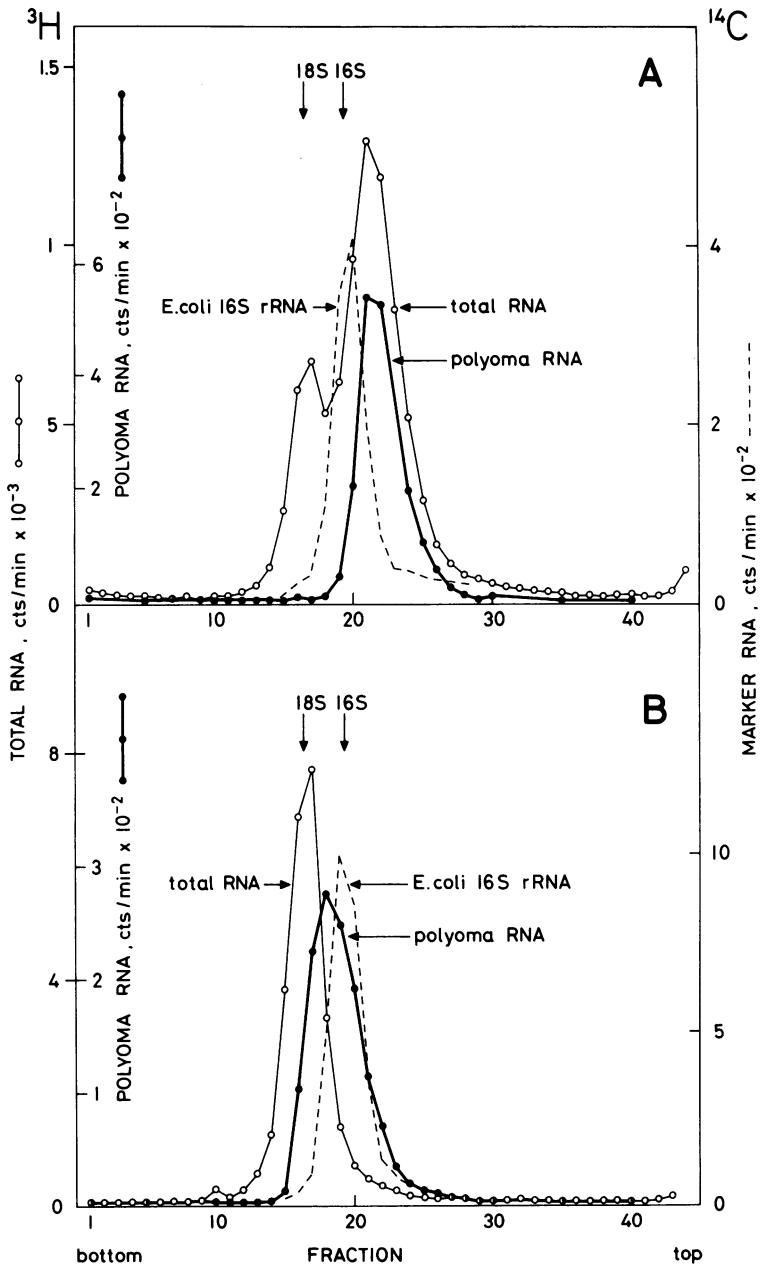

FIG. 7. *DMSO-sucrose gradient of 16S and 19S RNAs. From a sucrose gradient run in parallel to the one shown in Fig. 4 and containing RNA from the same experiment, the fractions corresponding to the 16S band (fractions 26 to 27) and the 19S shoulder (fractions 21 to 23) were separately pooled, as indicated by bars in Fig. 4. One-half of each RNA sample was precipitated in the presence of 10 μg of E. coli transfer RNA, resuspended, and mixed with approximately 0.5 and 1.5 μg, respectively, of ^{14}C-labeled E. coli 16S ribosomal RNA (about 3,000 counts per min per μg), in a total volume of 100 μliters of 90% DMSO, 1 mM EDTA (pH 7.2). The mixtures were then layered on two gradients made in 99% DMSO, 1 mM EDTA, containing 0 to 10% sucrose and 10 to 89% deuterated DMSO, and centrifuged in a Spinco SW 56 rotor (48,000 rpm, 8 h, 27 C). A sample of each fraction was counted ("total RNA" = total ^{3}H counts per minute per fraction, corrected for ^{14}C spill-over); the remainder was precipitated with ethanol in the presence of carrier E. coli transfer RNA, resuspended and hybridized to polyoma DNA ("polyoma RNA" = ^{3}H counts per minute hybridized per fraction). A, 16S pool; B, 19S pool.*

the 19S region forms a very broad band, which migrates even slower and thus presumably corresponds to the shoulder observed in Fig. 8. By superimposing the two patterns of separated 16 and 19S RNAs from Fig. 9A and B, one obtains a profile of viral RNA which is similar to that displayed by total, unfractionated RNA (Fig. 8).

In another experiment, polyribosomal RNA labeled with [^{3}H]uridine for 3 h (26 to 29 h after infection) was extracted and then sedimented through a DMSO gradient under the same conditions used in the experiment shown in Fig. 6. The fractions containing polyoma RNA were pooled, and the RNA was precipitated with ethanol in the presence of carrier *E. coli* transfer RNA, resuspended, and analyzed by polyacrylamide gel electrophoresis. In a parallel gel, untreated polyribosomal RNA was subjected to electrophoresis. The pattern of polyoma RNA collected from the DMSO gradient was virtually indistinguishable from that of untreated RNA and corresponded essentially to that shown in Fig. 8.

Polyribosomal RNA (labeled with [^{3}H]uridine between 26 and 29 h after infection) was also analyzed by electrophoresis in polyacrylamide gels in which formamide was used as a solvent instead of water, both in the gels and in the electrophoresis buffer. Although in our hands these gels did not allow a resolution as high as that obtained in aqueous gels, the profile of polyoma RNA consisted of a peak and a shoulder, similar to those observed in aqueous gels of comparable resolution. However, the peak of 16S polyoma RNA migrated in formamide gels slightly faster than 18S ribosomal RNA, in contrast to aqueous gels where it migrates slower than 18S RNA. This result is in agreement with the hypothesis that 16S, as well as 19S, polyoma RNA, undergoes conformational changes under denaturing conditions, like those of formamide-polyacrylamide gels. This was already suggested by the slight shift in sedimentation coefficient in DMSO gradients as compared to aqueous sucrose gradients.

In summary, these results show that polyoma RNA contains at least two distinct classes of molecules, which can be separated by sedimentation in aqueous sucrose gradients and by gel electrophoresis, and which seem to be able to undergo reversible conformational changes. It appears likely that conformational variations within 16 and 19S RNA molecules may, at least to some extent, account for the broadness of the bands formed by 16 and 19S RNAs in polyacrylamide gels. In addition, a true heterogeneity in molecular weights may play a role; the latter possibility is suggested by the broadness of the

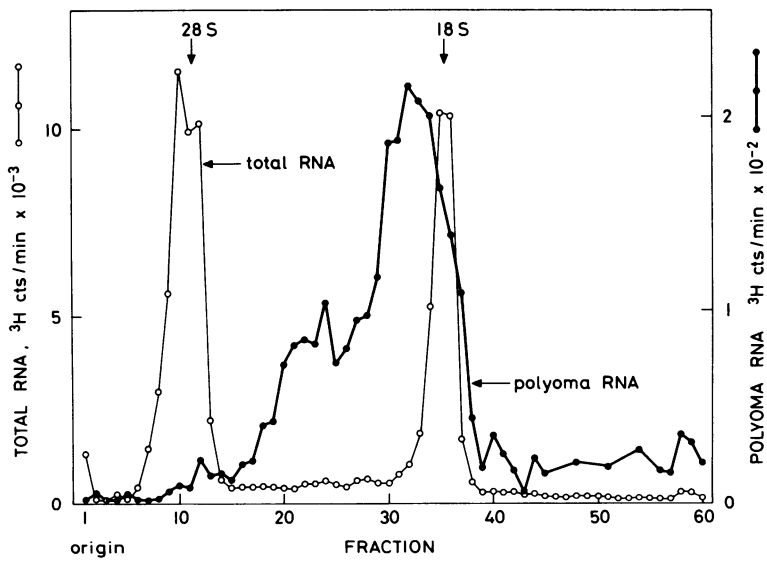

FIG. 8. *Gel electrophoretic analysis of polyribosomal RNA. Mouse kidney cell cultures were labeled with [^{3}H]uridine between 26 and 29 h after infection, and RNA was extracted from the isolated polyribosomes with phenol-chloroform-SDS at room temperature. About 8 μg of [^{3}H]RNA (16,000 counts per min per μg) was electrophoresed for 5.5 h. A sample of the eluate of each 2-mm slice was counted, and the remainder was hybridized to polyoma DNA on filters. "Total RNA" represents the total ^{3}H counts per minute per fraction, "polyoma RNA" the ^{3}H counts per minute per fraction hybridized to polyoma DNA. Blank filters contained less than 5 counts/min.*

band formed particularly by 19S RNA in DMSO gradients.

DISCUSSION

In the present work we labeled polyoma-infected mouse kidney cell cultures with [^{3}H]uridine for 3 h late in the lytic cycle (26 to 29 h after infection); we then analyzed polyoma-specific RNA extracted from cytoplasm and nuclei and from isolated polyribosomes by sedimentation in sucrose gradients, DMSO gradients and by electrophoresis on polyacrylamide gels.

The results reported here show that polyoma-

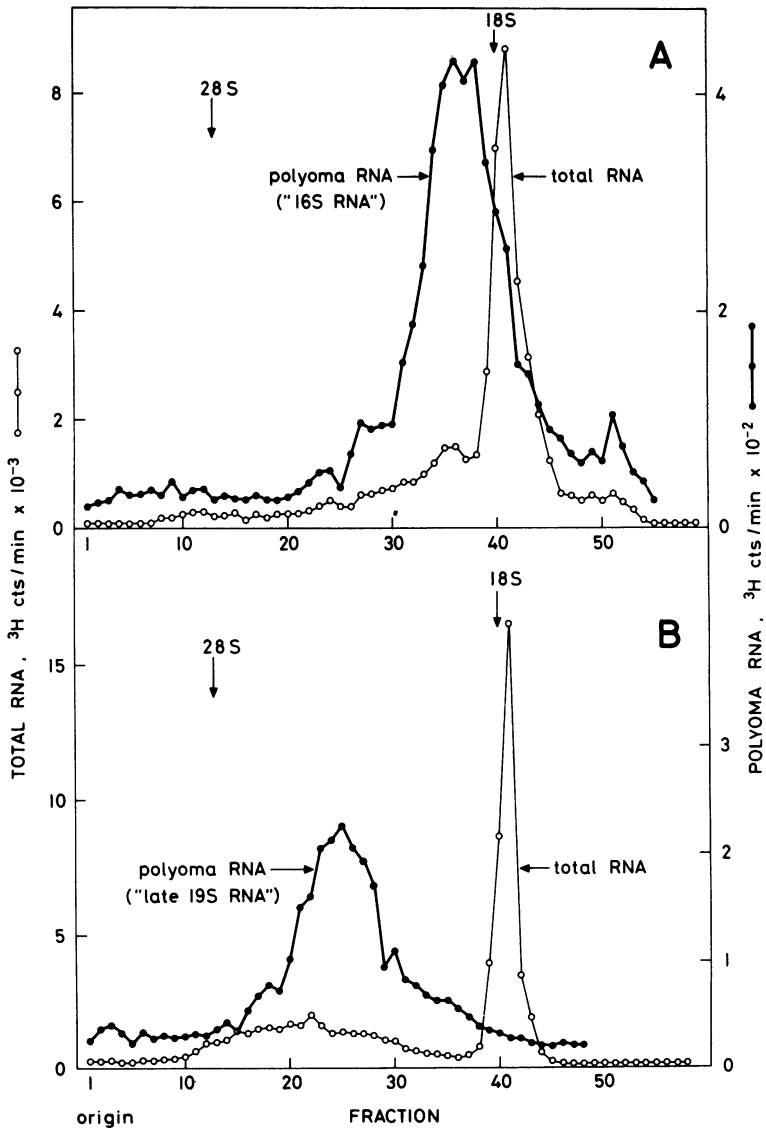

FIG. 9. *Gel electrophoretic analysis of 16 and 19S RNAs. From a sucrose gradient run in parallel to the one shown in Fig. 4 and containing RNA from the same experiment, the fractions corresponding to the 16S band (fractions 26 to 27) and to the 19S shoulder (fractions 21 to 23) were separately pooled. as indicated by bars in Fig. 4. The precipitated and resuspended RNA samples were mixed with ^{14}C-labeled mouse cell RNA and electrophoresed for 6 h. Total radioactivity of a sample of the eluate of each 2-mm slice was measured and the remainder was hybridized to polyoma DNA on filters. "Total RNA" = total ^{3}H counts per minute per fraction, corrected for ^{14}C spill-over. "Polyoma RNA" = ^{3}H counts per minute hybridized per fraction. The positions of 28 and 18S ribosomal RNAs (arrows) are derived from the profiles of the ^{14}C-marker RNA. A, 16S pool; B, 19S pool.*

specific RNAs with sedimentation coefficients of 26S or higher are exclusively present in the nucleus; in contrast, cytoplasmic viral RNA comprises two classes of RNA with sedimentation coefficients of 16S (major fraction) and 19S (minor fraction), as determined by sedimentation in sucrose gradients. This is at variance with other reports (6, 17) on the presence of large polyoma RNA (26 to 28S) in the cytoplasm of primary mouse kidney cell cultures late during the lytic infection.

It should be mentioned that in monkey kidney cell cultures undergoing a lytic infection with simian virus 40 (SV40), two bands (16 and 19S) of late SV40 RNA were detected by hybridization methods in RNA extracted from cytoplasm (27) and from whole cells (10). These results were obtained by sedimentation velocity analyses in aqueous sucrose gradients. In contrast, Tonegawa et al. (23), who analyzed cytoplasmic late SV40 RNA by sedimentation in 99% DMSO gradients, found a single band sedimenting slightly slower than 18S ribosomal RNA.

Our results show, furthermore, that both 16 and 19S polyoma RNAs are present in cytoplasmic polyribosomes in an association which is sensitive to treatment with EDTA; this suggests that they are mRNA molecules.

Polyoma-specific 16S RNA may contain the information for the synthesis of the major viral capsid protein; this is supported by the following indirect evidence. Synthesis of polyoma capsid protein is inhibited if polyoma-induced (cellular and viral) DNA synthesis is blocked with 5-fluorodeoxyuridine (FdU); after the synchronized onset of polyoma-induced DNA synthesis (which can be achieved by the release of the FdU block by addition of thymidine; 18), 16S polyoma RNA is synthesized, which is followed by the appearance of the major capsid protein (21). To obtain more direct evidence it will be necessary to test whether isolated 16S RNA is able to direct in vitro the synthesis of polyoma capsid protein.

Polyoma-specific late 19S RNA, the biological significance of which remains unknown, has a similar behavior in sucrose density gradients and in polyacrylamide gels to 19S polyoma RNA synthesized at late times after infection in the presence of FdU (26). It is not known whether the two contain the same species of polyoma RNA and whether they contain sequences transcribed from the same region as "early 19S RNA," which is synthesized in very small amounts from parental polyoma DNA at very early times after infection, and which is thought to carry the information for the "early" functions of polyoma virus (26).

Results reported in the present paper indicate that late polyoma RNA sediments, relative to ribosomal RNA, slightly more slowly in denaturing DMSO gradients than in ordinary sucrose gradients, the decrease in sedimentation coefficient being from 16 to 15S and from 19 to 17S. On the other hand, polyoma RNA migrates in polyacrylamide gels, with reference to ribosomal RNA, more slowly than expected from the sedimentation velocity analyses and from the results of electrophoresis in formamide gels. These observations can possibly be explained by (reversible) variations in the conformation of polyoma RNA under the different experimental conditions used for analysis. They may also account for the relative heterogeneity of the bands of polyoma RNA in polyacrylamide gels. The results presently available do not exclude the possibility that, in addition to structural heterogeneity, there may also exist a heterogeneity in molecular weights.

Assuming that the denaturing conditions in DMSO gradients allow a separation of RNA molecules based exclusively on chain length (this may not necessarily be true—see discussion in reference 7) and taking 28, 18S (11), and *E. coli* 16S (8) ribosomal RNAs as sedimentation markers, the molecular weights of 16 and 19S RNAs are estimated to be approximately 500,000 and 700,000, respectively. 16S RNA should then be able to code for a polypeptide with a molecular weight of about 50,000. The reported values for the molecular weight of the major viral capsid protein lie in this range (5, 19, 21).

Whereas Martin and Axelrod (9) showed that during the lytic cycle of polyoma one genome length of viral DNA is transcribed into stable RNA, the sum of our molecular weight estimates does not account for the equivalent of one genome. Therefore, these estimates may either be too low and will have to be revised, or the 16 and 19S RNA classes may contain more than one RNA species. For instance, the 16S RNA class could contain, in addition to the mRNA for the major capsid protein VP1, also the messenger for VP2 (molecular weight about 35,000). Fingerprint analyses of these proteins do not make clear at present whether VP2 is derived from VP1, as recently reported (3), or is a different protein (B. Hirt, personal communication). Our results do not exclude the existence of a distinct mRNA for VP2, particularly if present in low amounts, in which case it would not be resolved from the 16S RNA peak. A solution to this problem could be approached by using the separated strands of polyoma DNA or specific fragments of it in the hybridization assay across gradients or gels containing polyoma RNA.

ACKNOWLEDGMENTS

This work was done in the laboratory of Roger Weil, whom I wish to thank for encouragement and help in the preparation of this paper. I am particularly grateful to Nicholas H. Acheson who contributed stimulating discussions and advice. I would also like to thank Klaus Scherrer for continued interest in my work, Françoise Marti for excellent technical assistance, Otto Jenni for preparation of illustrations, George Mackie for a gift of ^{14}C-labeled *E. coli* 16*S* ribosomal RNA, and Peter Bromley and Pierre-François Spahr for critical reviews of the manuscript.

This work was supported by the Fonds National Suisse de la Recherche Scientifique, grants 3.276.69, 3.593.71 and 3.759.72.

This paper has been submitted to the Faculty of Science of the University of Geneva as part of a Ph.D. thesis.

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. Borun, T. W., M. D. Scharff, and E. Robbins. 1967. Preparation of mammalian polyribosomes with the detergent Nonidet P-40. Biochim. Biophys. Acta **149**:302-304.
3. Friedmann, T. 1974. Genetic economy of polyoma virus: capsid proteins are cleavage products of same viral gene. Proc. Nat. Acad. Sci. U.S.A. **71**:257-259.
4. Grollman, A. P. 1966. Structural basis for inhibition of protein synthesis by emetine and cycloheximide based on an analogy between ipecac alkaloids and glutarimide antibiotics. Proc. Nat. Acad. Sci. U.S.A. **56**:1867-1874.
5. Hirt, B., and R. F. Gesteland. 1971. Characterization of the proteins of SV40 and polyoma virus, p. 98-103. *In* L. G. Silvestri (ed.), The biology of oncogenic viruses. Second Lepetit Colloquium. North-Holland Publishing Company, Amsterdam.
6. Kajioka, R. 1972. Some studies on the status of polyoma-specified RNA after transcription. Virology **48**:284-287.
7. Kolakofsky, D., E. Boy de la Tour, and H. Delius. 1974. Molecular weight determination of Sendai and Newcastle disease virus RNA. J. Virol. **13**:261-268.
8. Kurland, C. G. 1960. Molecular characterization of ribonucleic acid from Escherichia coli ribosomes. J. Mol. Biol. **2**:83-91.
9. Martin, M. A., and D. Axelrod. 1969. Polyoma virus gene activity during lytic infection and in transformed animal cells. Science **164**:68-70.
10. May, E., P. May, and R. Weil. 1973. "Early" virus-specific RNA may contain information necessary for chromosome replication and mitosis induced by simian virus 40. Proc. Nat. Acad. Sci. U.S.A. **70**:1654-1658.
11. McConkey, E. H., and J. W. Hopkins. 1969. Molecular weights of some HeLa ribosomal RNA's. J. Mol. Biol. **39**:545-550.
12. Mirault, M.-E., K. Scherrer, and L. Hansen. 1971. Isolation of preribosomes from HeLa cells and their characterization by electrophoresis on uniform and exponential-gradient-polyacrylamide gels. Eur. J. Biochem. **23**:372-386.
13. Pène, J. J., E. Knight, Jr., and J. E. Darnell, Jr. 1968. Characterization of a new low molecular weight RNA in HeLa cell ribosomes. J. Mol. Biol. **33**:609-623.
14. Penman, S., C. Vesco, and M. Penman. 1968. Localization and kinetics of formation of nuclear heterogeneous RNA, cytoplasmic heterodisperse RNA and polyribosome-associated messenger RNA in HeLa cells. J. Mol. Biol. **34**:49-69.
15. Perry, R. P., and D. E. Kelley. 1968. Messenger RNA-protein complexes and newly synthesized ribosomal subunits: analysis of free particles and components of polyribosomes. J. Mol. Biol. **35**:37-59.
16. Perry, R. P., J. La Torre, D. E. Kelley, and J. R. Greenberg. 1972. On the lability of poly(A) sequences during extraction of messenger RNA from polyribosomes. Biochim. Biophys. Acta **262**:220-226.
17. Petric, M., and J. B. Hudson. 1972. The processing of polyoma virus RNA synthesized during productive infection in mouse cells. Can. J. Biochem. **50**:927-935.
18. Pétursson, G., and R. Weil. 1968. A study on the mechanism of polyoma-induced activation of the cellular DNA-synthesizing apparatus. Arch. Gesamte Virusforsch. **24**:1-29.
19. Roblin, R., E. Härle, and R. Dulbecco. 1971. Polyoma virus proteins. I. Multiple virion components. Virology **45**:555-566.
20. Scherrer, K. 1969. Isolation and sucrose gradient analysis of RNA, p. 413-432. *In* K. Habel and N. P. Salzman (ed.), Fundamental techniques in virology. Academic Press Inc., New York.
21. Seehafer, J. G., and R. Weil. 1974. Synthesis of polyoma virus structural polypeptides in mouse kidney cells. Virology **58**:75-85.
22. Strauss, J. H., Jr., R. B. Kelly, and R. L. Sinsheimer. 1968. Denaturation of RNA with dimethylsulfoxide. Biopolymers **6**:793-807.
23. Tonegawa, S., G. Walter, A. Bernardini, and R. Dulbecco. 1970. Transcription of the SV40 genome in transformed cells and during the lytic infection. Cold Spring Harbor Symp. Quant. Biol. **35**:823-831.
24. Vaughan, M. H., J. R. Warner, and J. E. Darnell. 1967. Ribosomal precursor particles in the HeLa cell nucleus. J. Mol. Biol. **25**:235-251.
25. Weil, R., and J. Kára. 1970. Polyoma "Tumor antigen": an activator of chromosome replication? Proc. Nat. Acad. Sci. U.S.A. **67**:1011-1017.
26. Weil, R., C. Salomon, E. May, and P. May. A new look at the mode of action of polyoma and related tumor viruses. Presented at the Second International Conference on Comparative Virology, Mont Gabriel, Canada, August 1973.
27. Weinberg, R. A., S. O. Warnaar, and E. Winocour. 1972. Isolation and characterization of simian virus 40 ribonucleic acid. J. Virol. **10**:193-201.
28. Winocour, E. 1963. Purification of polyoma virus. Virology **19**:158-168.