RNAs of simian virus 40 in productively infected monkey cells: Kinetics of formation and decay in enucleate cells*

(cytochalasin B/cytoplasmic RNA/RNA processing/sucrose gradients/hybridization)

YOSEF ALONI, MOSHE SHANI, AND YAFFA REUVENI

Department of Genetics, The Weizmann Institute of Science, Rehovot, Israel

Communicated by Norman Davidson, April 21, 1975

ABSTRACT   We demonstrate here the usefulness of cyto-chalasin B enucleate cells for the study of the metabolism of cytoplasmic mRNA and for determining its half-life in ani-mal cells. Simian virus 40 infected monkey cells in which the RNA had been labeled with [³H]uridine were enucleated, and the decay of the two prominent RNAs of simian virus 40, the 19S and 16S species, was measured by analysis on sucrose gradients. The results of these experiments, together with ki-netic analysis of nuclear and cytoplasmic viral RNA, indicate a precursor–product relationship between the 19S and 16S cytoplasmic viral RNA species, which decay by first-order ki-netics with a mean half-life of about 3 hr and 6 hr, respec-tively.

A crucial parameter in any model for protein synthesis is the lifetime of messenger RNA (1). Previous studies on the life-time of RNA in animal cells using either the conventional pulse-chase technique or actinomycin D have yielded incon-clusive results (2). Recently, these studies have been greatly facilitated by the development of improved chase conditions (3) and by the discovery that a large percentage of mamma-lian mRNA contains a poly(A) segment (4–6). These meth-ods have demonstrated that most mRNA molecules decay by first-order kinetics and fall into two classes, in one of which the molecules have an average half-life of 7 hr and in the other of one cell generation (3, 7, 8). In the present work, we demonstrate the usefulness of enucleate cells, as an addition-al system for the study of cytoplasmic mRNA stability and processing. Enucleates have an advantage over intact cells since the superimposed complexity of the transcription pro-cess in the nucleus is eliminated. The normality of these cy-toplasms is rather surprising (9, 10), as indicated by criteria such as cell movement, ability to allow virus replication, and protein synthesis (10, 11). Enucleate monkey cells infected with simian virus 40 (SV40) were used here to study the re-lationship between the cytoplasmic viral RNA species and their relative metabolic stability.

SV40 gives a productive, lytic infection in monkey kidney cells. During the late phase of the lytic cycle, two prominent cytoplasmic viral RNA species of about 900,000 and 600,000 daltons are observed which sediment on sucrose gradient as 19 S and 16 S, respectively (12). The larger late 19S species shares nucleotide sequences with, and is less stable than, the smaller 16S component (13). It will be shown that the 19S and 16S viral RNA components exhibit a precursor–product relationship and that they decay by first-order kinetics with a mean half-life of about 3 hr and 6 hr, respectively.

Abbreviations: NaDodSO₄, sodium dodecyl sulfate; SV40, simian virus 40.
* This is article III in the series "Transcription of SV40." Paper I is ref. 22 and paper II is ref. 20.

MATERIALS AND METHODS

Growth of plaque-purified SV40 (strain 777) on monkey cells of the BSC-1 line, as well as concentration and purifica-tion of the virus from the tissue culture lysates and prepara-tion of SV40 DNA component I, have been described (14). In all experiments, cultures (4 × 10⁶ cells) were infected with 1.0 ml of the same stock of SV40 [2 × 10⁸ plaque-form-ing units/ml]. For enucleation of the infected cells, plastic 22 mm diameter "coverslips" were cut from the bottom of Falcon tissue culture plates (9) with a cork borer. The "cov-erslips" were placed in 50-ml Sorvall plastic centrifuge tubes containing 10 μg/ml of cytochalasin B in 5 ml of Eagle's medium with a 4-fold concentration of amino acids and vi-tamins supplemented with 2% calf serum, such that the side with the cells faced downwards. The tubes were placed in a Sorvall SS-34 head seated in a Sorvall RC-2 centrifuge that had been warmed to 37°. The tubes were centrifuged at 12,000 rpm for 15 min at 37°. After centrifugation, the "coverslips" were removed from the tubes and placed in tis-sue culture dishes containing normal medium in order to re-cover from the effect of cytochalasin B. Within 30–60 min the cells possessed a normal morphology. About 95% of the cells were enucleated.

For the preparation of ³H-labeled RNA, the infected cul-tures were washed with phosphate-buffered saline (15) and treated with Nonidet P-40 detergent to give nuclear or cyto-plasmic fractions (16) or with cytochalasin B to give enucle-ate cells (9). RNA was extracted as described (16). All RNA samples were digested with 50 μg/ml of DNase I (Worth-ington, RNase-free electrophoretically purified). The digest was extracted with sodium dodecyl sulfate (NaDodSO₄)/ phenol and fractionated on sucrose gradients (17).

RNA·DNA hybridization with the DNA immobilized on Millipore filters (25 mm diameter, 0.45 μm pore size) and selection of the viral RNA by preparative hybridization in 50% formamide were described (12, 17).

RESULTS

Sedimentation Patterns of Pulse-Labeled Viral RNA Ex-tracted from Nuclei of SV40-Infected BSC-1 Cells. In la-beling experiments, the extent to which precise kinetic mea-surements of minor species such as viral RNA in infected cells can be made depends on the ability to identify the minor species in the presence of the more abundant species or to isolate the minor species from the more abundant species. Accordingly, we have used a combination of sucrose gradient centrifugation and RNA·DNA hybridization to dis-tinguish the labeled SV40 RNA from the background of cel-lular RNA (14, 18).
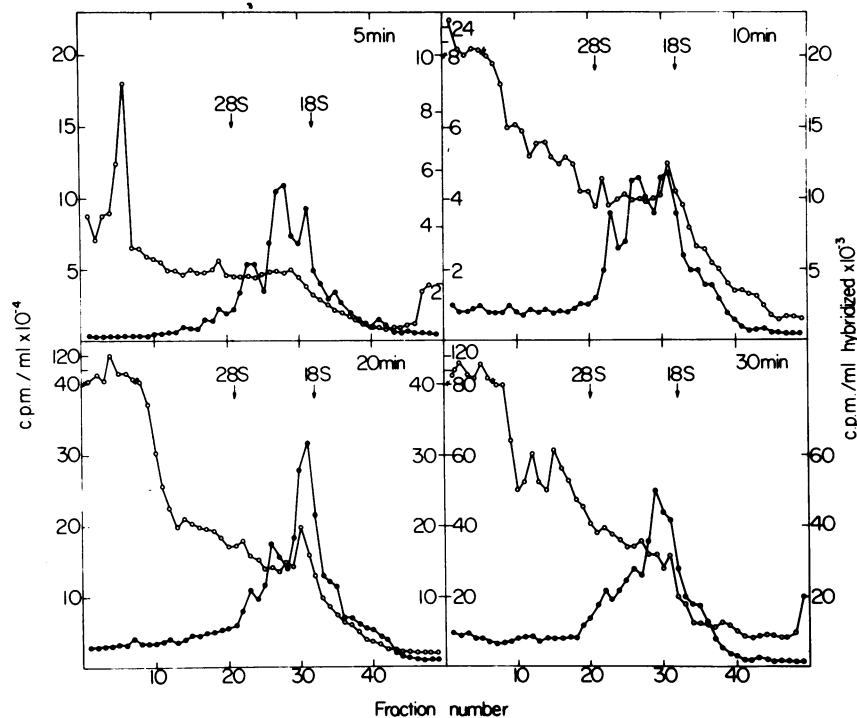
Froction number

FIG. 1.   Pulse-labeled viral RNA in nuclei of infected cells. Forty-eight hours after infection, 3×10^7 BSC-1 cells were labeled with [5,6-^{3}H]uridine (0.1 mCi/ml, 48 Ci/mmol). At the indicated times, incorporation was stopped by placing the petri dishes on ice and removing the medium. The cells were washed four times with phosphate/saline (15) and lysed with Nonidet P-40 detergent (16). The nuclei were digested with electrophoretically purified DNase, and the RNA was extracted with NaDodSO$_4$/phenol (17). [^{3}H]RNA was placed on linear gradients of 15–30% (w/w) sucrose in NaDodSO$_4$ buffer (26). Centrifugation was for 16 hr at 25,000 rpm at 20° in a Spinco SW 27 rotor. The radioactivity of 50-μl aliquots of each fraction was determined (22). *Arrows* indicate the positions of ^{32}P-labeled 28S and 18S rRNA markers. Aliquots (50-μl each) from each fraction of the gradient were incubated in 0.4 ml of 0.60 M NaCl/0.06 M Na$_3$ citrate with 7-mm filters containing 0.5 μg of SV40 DNA for 18 hr at 68°. The filters were then treated with RNase; blank filters had less than 5 cpm. (Similar profiles were obtained when the incubation was with 25 μl from each fraction.) The results are shown as cpm/ml. With RNA extracted from mock-infected cells, less than 7 cpm in any one fraction hybridized with SV40 DNA (pattern not shown). O, cpm; ●, cpm hybridized.

Forty-eight hours after infection with SV40, BSC-1 cells were exposed to [^{3}H]uridine for various lengths of time, and RNA was extracted from the nuclei and subjected to sucrose gradient analysis. Fig. 1 shows that the bulk of the *cellular* labeled RNA in the nucleus is found as high-molecular-weight polydisperse species present in a broad band heavier than the 28S ribosomal RNA marker. Similar polydisperse RNA has been found in other cells (16). About 20% of the *viral* RNA sediments as fast as the polydisperse species; but the great majority of the virus-specific RNA is distributed with several reproducible peaks between the 28S and 18S rRNA markers. Essentially none of the SV40 specific RNA sediments slower than 18S ribosomal RNA (0.7 × 10⁶ dalton) (19), suggesting that no extensive degradation occurred during extraction and analysis of the RNA. The sharpness and reproducibility of some of the peaks preclude the possibility that they resulted from random degradation. Molecular weight estimates of the viral RNA suggest that the first recognizable viral RNA peak (26S) in the nucleus is compatible with a complete SV40 RNA transcript (about 1.5 × 10⁶ dalton). About 90% of the labeled RNA present in this peak is complementary to the "late strand" (+ strand), and 10% is complementary to the "early strand" (−strand) (Laub and Aloni, in preparation). If the molecules present in the 26S peak are indeed the RNA transcripts of the entire SV40 DNA molecule, this would mean that SV40 DNA is transcribed symmetrically over its entire length as was suggested (20). Yet the two types of RNA transcripts differ in their

proportion; the "late" RNA transcripts are 10 times more abundant in the nucleus than the "early" RNA transcripts. The latter presumably reflects the rate of formation of the two RNA transcripts rather than their relative metabolic stability (Laub and Aloni, in preparation). A second, broader peak sediments at about 23–24 S and appears to be composed of more than one species of RNA, and a third peak sediments at about 19 S. No 16S viral RNA peak has been detected in the nucleus of infected cells labeled for up to 30 min with [^{3}H]uridine. With the increase in labeling time from 5 to 30 min, more radioactivity is incorporated into the 19S species compared to the incorporation of radioactivity into the 26S and 23–24S peaks, suggesting that the larger species are more labile than the 19S species. It is suggested that the RNA molecules present in the 26S peak are the primary viral RNA transcripts and the RNA molecules present in the 19S peak are the *final* products of the processing of the viral RNA in the nucleus. Since 16S RNA is the major viral component in the cytoplasm and is not recognizable in the nucleus, it appears that at least part of the molecules present in the 19S peak are further processed in the cytoplasm. Alternatively, it could be that other short-lived RNA molecules are transported to the cytoplasm and they serve as the immediate precursors to the mature viral RNA species.

Sedimentation Patterns of Labeled Viral RNA Extracted from Cytoplasm of SV40-Infected Cells. The specific activities of the mature viral RNAs extracted from the cytoplasm of infected cells exposed for short labeling time is low,

Biochemistry: Aloni *et al.*

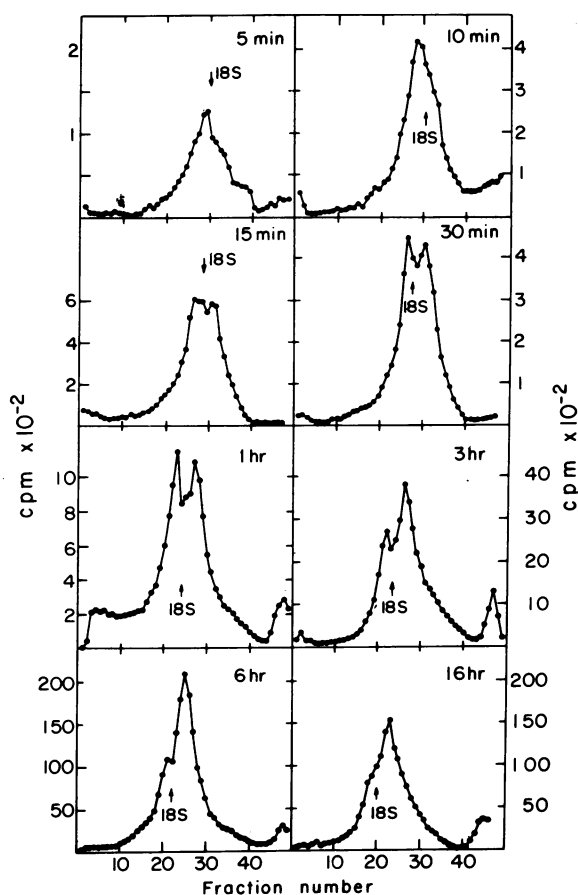*Proc. Nat. Acad. Sci. USA 72 (1975)* 2589

FIG. 2. Pulse-labeled viral RNA in the cytoplasm of infected cells. BSC-1 cells were infected with SV40 and labeled, and the cytoplasmic fraction was prepared with Nonidet P-40 detergent (16). [^{3}H]RNAs were extracted with NaDodSO$_4$/phenol, treated with DNase (27), and were then centrifuged through sucrose gradients to determine if there is contamination by nuclear RNA (16) and also to remove the digested DNA. Fractions sedimenting between 8 and 35S were pooled, and viral RNA sequences were isolated by hybridizing to and eluting from filters containing 20 μg of SV40 DNA (12). (About 10% of the input radioactive RNA was bound to and eluted from SV40 DNA filters, and less than 0.1% of the radioactivity was bound to blank filters.) The viral selected [^{3}H]RNAs were then centrifuged through 15–30% (w/w) sucrose gradients (in a Spinco SW-27 rotor for 21 hr at 25,000 rpm at 20°). Fractions were collected, and radioactivity was determined in Triton X-100 scintillation fluid (22). *Arrow* indicates the position of ^{32}P-labeled 18S rRNA marker.

and a large amount of viral DNA is needed to identify them. We, therefore, preferred to use the hybridization/elution technique in formamide with excess DNA for isolating these cytoplasmic viral RNA species (12). Fig. 2 shows that 19S viral RNA appears in the cytoplasm after a 5-min pulse, and it is the predominant species for up to about 60 min of labeling. Moreover, the 19S species is a homogeneous "late" RNA transcript, as indicated by the finding that less than 2% of this component is complementary to the "early" strand (Laub and Aloni, in preparation). The 16S viral RNA is first seen as a distinct peak after a 15-min pulse, and it increases in proportion with the length of the pulse. Thus, equal amounts of 16S and 19S RNA are seen after about a 1-hr pulse, while after a 16-hr labeling period, about 90% of the labeled viral RNA is found in the 16S peak. Similar results were obtained when the cytoplasmic RNA was first sedi-

mented through a gradient and then each fraction was hybridized with excess amounts of SV40 DNA (22). However, with the latter technique, the labeled 19S species predominate for longer durations, and the 1:1 ratio between the two peaks was obtained after the cells were labeled for 4–5 hr. The lower 19S/16S ratio obtained by the selection/hybridization technique may arise from a mild degradation of some of the 19S molecules during the selection step so that the broken 19S molecules sediment in the 16S peak, and/or it may result from competition between their common sequences (13) and the 16S sequences that are in excess. These results, together with the data from the analysis of the viral RNA in the nucleus, indicate that the change in the proportion of the 19S and 16S RNAs takes place after they have entered the cytoplasm; the larger, late RNA is metabolically more labile than the smaller one.

Formation and Decay of Viral RNA Species in Enucleate Cells. SV40-infected cells were labeled with [^{3}H]uridine for 3 hr at 48 hr after infection. Nuclei were removed with cytochalasin B (9), and the enucleate cells were refed with fresh medium and allowed to recover for 1 hr. At this time, it was observed that the elongated thin structures seen immediately after enucleation tended to regain the shape of the original cells but without nuclei. The contamination with nucleated cells, as determined by staining the cells with Giemsa and counting intact and enucleate cells, was always less than 5%. "Coverslips" containing 0.5 × 10^6 cells were removed at various times, and the RNA was extracted with phenol and NaDodSO$_4$ prior to sucrose gradient analysis. Fig. 3 shows the sedimentation patterns of these RNA samples. The 28S and 18S RNAs provide convenient sedimentation markers, serve as standards for the amount of RNA in the preparations, and serve to estimate the level of contamination with nucleate cells. The level of contamination is indeed low, as seen from the only slight increase of total radioactivity incorporated into the stable 28S and 18S rRNAs and the only small increase in the 28S/18S ratio during 16 hr of incubation (16).

The profiles of SV40-specific RNA were determined from the radioactivity remaining bound to filters after the labeled RNA present in the indicated fractions of the sucrose gradient was hybridized with an excess of SV40 DNA and treated with RNase (22). Fig. 3 shows that late lytic RNA consists of two prominent species of RNA, as was reported previously (12). After a 3-hr labeling period, a profile of late viral RNA shows the 19S species with a peak height of about 1.2 times that of the peak of the smaller RNA species. A comparison between the various time intervals indicates the rapid decay of the labeled viral RNA components relative to the labeled ribosomal RNAs. Furthermore, the height of the 19S peak decreases immediately, while that of the 16S peak even increases slightly at early times after incubation of the labeled enucleates and starts to decrease only after 5–6 hr. At this time, the 19S peak is only 20–25% of its original height.

Fig. 4 summarizes the results of two experiments as shown in Fig. 3, measuring the rate of decay of 19S and 16S viral RNAs. It appears that the decay rate of the 19S component approximates first-order kinetics, with a mean half-life of about 3 hr. Analysis of the data for the 16S component leads to a decay curve that can be divided into two periods; up to 5–6 hr, there is a slight increase in the radioactivity incorporated into the 16S component, and only then does decay proceed exponentially with a half-life of about 6 hr. Since there is no net viral RNA synthesis in the enucleate cells, and no other recognizable viral RNA components in the cytoplasm
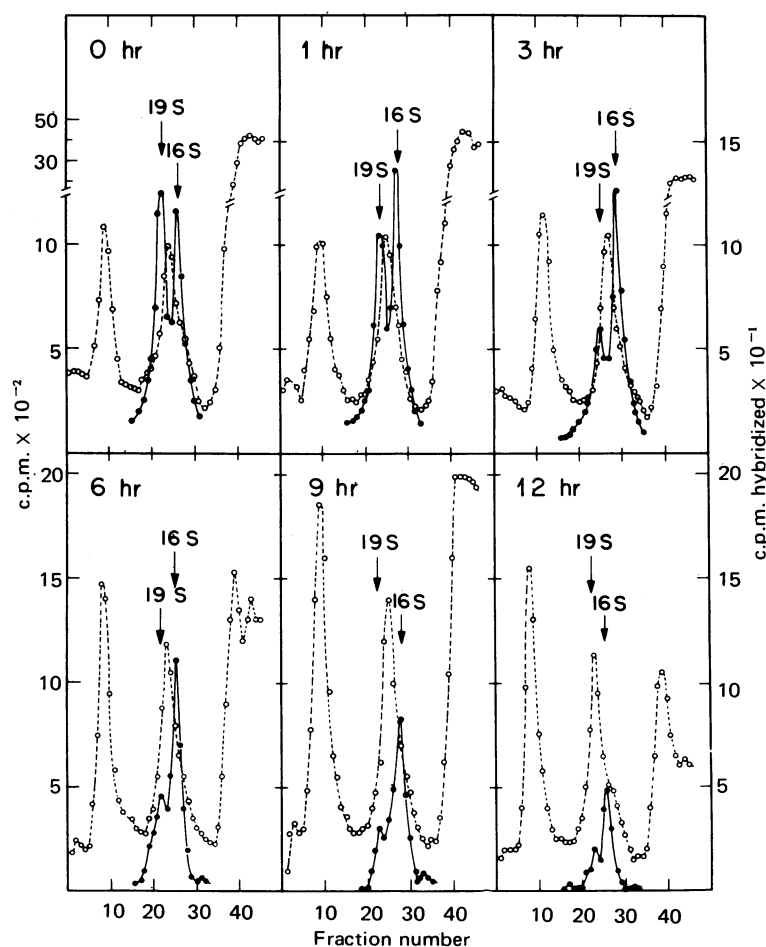
FIG. 3. Viral [³H]RNAs in enucleate SV40-infected cells. BSC-1 cells were infected with SV40 and labeled for 3 hr with [5,6-³H]uridine (0.1 mCi/ml, 48 Ci/mmol) at 48 hr after infection. Enucleate cells were prepared with cytochalasin B (9) (see *Materials and Methods*) and allowed to recover for 1 hr (zero time). At intervals afterwards the same number of "coverslips" were removed and [³H]RNA was extracted with NaDodSO₄/phenol, treated with DNase (20), and centrifuged in 15–30% (w/w) sucrose gradients in NaDodSO₄ buffer (26). Centrifugation was for 20 hr at 25,000 rpm at 20° in a Spinco SW27 rotor; the radioactivity of 50-μl aliquots was determined directly in Triton X-100 scintillation fluid (22) (O). The two peaks are those of the 28S and 18S rRNAs (22). Aliquots (150 μl each) from the fractions of the gradients that contain the prominent viral RNAs species (12) were incubated in 0.4 ml of 0.60 M NaCl/0.06 M Na₃ citrate with 7-mm filters containing 0.5 μg of SV40 DNA for 20 hr at 68°. The cpm hybridized represents the radioactivity bound to the filters after treatment with RNase (22) (●). Blank filters had less than 5 cpm.
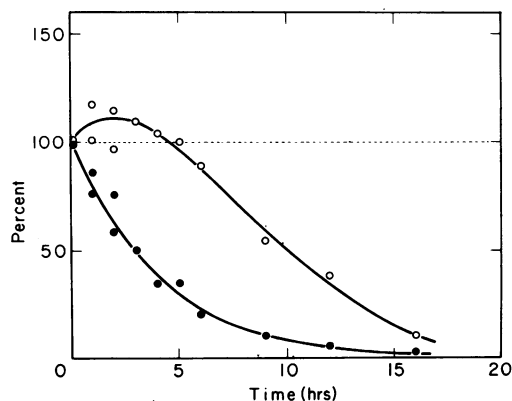

FIG. 4. Decay of the prominent viral RNA species in enucleate cells. The data are from sucrose gradient analysis of two experiments such as shown in Fig. 3. Calculation of the area of the 16S and 19S peak is difficult due to overlap and uncertainty of the baseline. To avoid this problem, the sum of the cpm present in the peak fraction and the three points to the left of the 19S peak was taken as an estimate of the amount of radioactivity in the 19S

(12), it is suggested that the initial increase of radioactivity found in the 16S peak originates from a portion of the 19S molecule that shares common base-sequences with the 16S component (13). Interpretation of these results leads to the conclusion that the 19S and 16S viral RNA components exhibit a precursor–product relationship.

DISCUSSION

The results presented here demonstrate the usefulness of enucleate cells for the study of the metabolism of cytoplasmic mRNA and for determination of the mean half-life of mRNA in animal cells. Studies with enucleate cells have an advantage over studies of intact cells, since the superim-

species. Similarly, the 16S component was estimated from the sum of the cpm in the peak fraction and the three points to the right of it. These estimates were than normalized by integration of the ³H-labeled ribosomal RNAs. The estimated radioactivity for each RNA component at zero time was taken as 100%. ●, 19S RNA; O, 16S RNA.

Biochemistry: Aloni et al.

Proc. Nat. Acad. Sci. USA 72 (1975)   2591

posed complexity of the transcription process in the nucleus is eliminated; thus, they allow direct determination of mRNA half-life. It appears that besides the loss of nuclear functions, the enucleate cells are functionally normal, as indicated by criteria such as cell movement and ability to allow viral replication and to support protein synthesis (9–11). Furthermore, there seems to be no damage to the RNA metabolism, since rRNA is as stable in the enucleate cells as in nucleate cells. It is assumed, therefore, that any decay of other RNA molecules in enucleate cells reflects the situation in normal cells; however, this assumption must be confirmed.

In the present work enucleate, SV40-infected cells were used to study the relationship between the prominent viral RNAs, the cytoplasmic 19S and 16S RNA species, and their relative metabolic stabilities.

From kinetic analysis of the viral RNAs in the nucleus and in the cytoplasm of infected cells, it appears (Figs. 1 and 2) that the 19S viral RNA species is recognizable in the nucleus while the 16S peak is not. Furthermore, labeled 19S "late" transcripts appear in the cytoplasmic fraction after a 5-min pulse, while the 16S component is found in the cytoplasmic fraction as a distinct peak only after a 15-min pulse and it increases in proportion with the length of the pulse to become the major labeled viral RNA component. This, together with previous finding that the 19S and 16S RNA components hybridize to the same SV40 DNA fragments and therefore share common base sequences (13), suggests that the 19S RNA molecules contain the precursor to the 16S RNA molecules. The final evidence that the two prominent viral RNA species exhibit a precursor–product relationship comes from their behavior in enucleate cells (Figs. 3 and 4). It appears that whereas the 19S RNA component decays exponentially with approximately first-order kinetics with a mean half-life of about 3 hr, there is an initial increase in radioactivity incorporated into the 16S component, and only 5–6 hr later, when the 19S species has already decayed to 20–25% of its original cpm, an exponential decay curve is found also for the 16S component. Since there is no net viral RNA synthesis in the enucleate cells and no other distinct viral RNA component in the cytoplasm (12), it is suggested that the initial increase in radioactivity found in the 16S component originates from the 19S RNA species. Thus, the following conclusions emerge from this and previous work: (i) the 19S and 16S RNA species decay exponentially; (ii) ⅔ of the sequences present in the 19S molecule (molecular weight 900,000) are processed to the 16S RNA molecule (molecular weight 600,000); and (iii) there are Gaussian distributions around the peak fractions. Based on these conclusions and on the assumption that enucleation does not affect mRNA stability, the best fit approximation of the observed data (computer-derived) leads to half-life values for the 19S and 16S RNA components of about 3 hr and 6 hr, respectively. The demonstrated cytoplasmic conversion of the 19S species to 16S component in enucleate cells provides a unique system for studying cytoplasmic processing of nuclear mRNA transcripts.

Two of the immediate questions rising from this report

deal with the events that lead to the cleavage of the 19S species to the 16S component, and with the nature of the recognition sites for the endo- or/and exonuclease on the 19S molecule. Concerning the first question, it could be suggested that the 19S RNA is first complexed in polysomes and only then can it be processed to the 16S RNA. It has been shown (25) that the 19S RNA directs the synthesis in a cell-free system of a product ("x" polypeptide) that is present in infected cells and not in virions, and it has been suggested (25) that at this stage the initiation site for the major viral polypeptide VP-1 is inaccessible. Only after the 19S RNA is cleaved, can the 16S RNA be translated to VP-1. Concerning the signal for the cleavage site, it could be either sequence-specific or added at a post-transcriptional stage.

1. Levintal, C., Keynan, A. & Higa, A. (1962) Proc. Nat. Acad. Sci. USA 48, 1631–1638.
2. Singer, R. H. & Penman, S. (1972) Nature 240, 100–102.
3. Murphy, W. & Attardi, G. (1973) Proc. Nat. Acad. Sci. USA 70, 115–119.
4. Edmonds, M., Vaughan, M. & Nakagato, H. (1971) Proc. Nat. Acad. Sci. USA 68, 1336–1340.
5. Lee, S. Y., Mendecki, J. & Brawerman, G. (1971) Proc. Nat. Acad. Sci. USA 68, 1331–1335.
6. Darnell, J. E., Wall, R. & Tushinski, R. S. (1971) Proc. Nat. Acad. Sci. USA 68, 1321–1325.
7. Perry, R. P. & Kelley, D. E. (1973) J. Mol. Biol. 79, 681–696.
8. Singer, R. H. & Penman, S. (1973) J. Mol. Biol. 78, 321–334.
9. Prescott, D., Myerson, D. & Wallace, J. (1972) Exp. Cell Res. 71, 480–485.
10. Goldman, R. D., Pollack, R. & Hopkins, N. H. (1973) Proc. Nat. Acad. Sci. USA 70, 750–754.
11. Pollack, R. & Goldman, R. (1973) Science 179, 915–916.
12. Weinberg, R. A., Warnaar, S. O. & Winocour, E. (1972) J. Virol. 10, 193–201.
13. Weinberg, R. A., Ben-Ishai, Z. & Newbold, J. E. (1974) J. Virol. 13, 1263–1273.
14. Aloni, Y., Winocour, E. & Sachs, L. (1968) J. Mol. Biol. 31, 415–429.
15. Dulbecco, R. & Vogt, M. (1954) J. Exp. Med. 99, 167–182.
16. Penman, S. (1966) J. Mol. Biol. 17, 117–130.
17. Aloni, Y. & Attardi, G. (1971) J. Mol. Biol. 55, 251–270.
18. Gillespie, D. & Spiegelman, S. (1965) J. Mol. Biol. 12, 829–842.
19. McConkey, E. H. & Hopkins, J. W. (1969) J. Mol. Biol. 39, 545–550.
20. Aloni, Y. (1973) Nature New Biol. 243, 2–6.
21. Adensnik, M. & Darnell, J. E. (1972) J. Mol. Biol. 67, 397–406.
22. Aloni, Y. (1972) Proc. Nat. Acad. Sci. USA 69, 2404–2409.
23. Emerson, C. P., Jr. (1971) Nature New Biol. 232, 101–106.
24. Abelson, H. T., Johnson, L. F., Penman, S. & Green, H. (1974) Cell 1, 161–165.
25. Prives, C. L., Aviv, H., Gilboa, E., Revel, M. & Winocour, E. (1974) Cold Spring Harbor Symp. Quant. Biol., in press.
26. Aloni, Y. & Attardi, G. (1972) J. Mol. Biol. 70, 375–381.
27. Aloni, Y. & Locker, H. (1973) Virology 54, 495–505.