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Transcription of the Polyoma Virus Genome: Synthesis and Cleavage of Giant Late Polyoma-Specific RNA

(gel electrophoresis/density gradient centrifugation/RNA–DNA hybridization/tumor virus)

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ABSTRACT The size of virus-specific RNA synthesized in cultured mouse kidney cells infected with polyoma virus was estimated by electrophoresis and sedimentation analysis of RNA extracts from whole cells. Newly synthesized "late" polyoma-specific RNA appears as "giant" molecules of heterogeneous size, up to several times larger than a strand of polyoma DNA (1.5×10^6 daltons). Treatment with dimethylsulfoxide or urea showed that the large size of these molecules is not due to aggregation. Giant polyoma-specific RNA is strikingly similar in size distribution to "nuclear messenger-like" RNA ("heterogeneous nuclear" RNA) of the host cell. Subsequent to its synthesis, some of the giant polyoma-specific RNA appears to be cleaved to at least three smaller species.

During lytic infection of mouse kidney cell cultures with polyoma virus, virus-specific RNA can be detected by hybridization with polyoma DNA (1, 2). Viral RNA transcribed after the onset of polyoma DNA replication has been designated as "late" RNA (2). The rate of synthesis of late RNA increases rapidly beyond 12 hr after infection and approaches a maximum at about 30 hr.

Earlier experimental results suggested that late polyoma-specific RNA is the transcript of most or all of the genetic information contained in a strand of polyoma DNA (2). We undertook the present experiments to define more precisely the size of late virus-specific RNA found in polyoma-infected cells. Total RNA was extracted about 30 hr after infection from [^{3}H]uridine-labeled cultures under conditions that minimize breakdown, and was analyzed by gel electrophoresis and sucrose or Me_2SO–sucrose gradient centrifugation. RNA from each gel or gradient fraction was hybridized with an excess of polyoma DNA to determine the amount of virus-specific RNA present. The results show that the bulk of late polyoma-specific RNA is synthesized as "giant" molecules larger than the viral genome, and that some of this giant RNA is subsequently cleaved to smaller RNAs of specific sizes.

MATERIALS AND METHODS

Preparation of ^{3}H-labeled RNA

Primary mouse kidney cell cultures (3) grown in 88-mm Petri dishes were infected (4) with wild-type polyoma virus 1 or 2 days after confluence. Cells were pulse-labeled for 20 min at 37°C with 500 μCi per dish of [5-^{3}H]uridine (New England Nuclear Corp., 25 Ci/mmol) in 1 ml of warm reinforced Eagle's medium, scraped into ice-cold, isotonic, phosphate-buffered saline, and lysed within 5–10 min by dilution into 0.01 M sodium acetate (pH 5.1)–1% sodium dodecyl sulfate. Within 10–30 sec of lysis, total cellular RNA was extracted with redistilled phenol; this treatment was repeated twice, for a total of 8 min at 60–65°C (5). Nucleic acids were precipitated with ethanol, redissolved, and incubated at 0°C for 30 min with 10 μg/ml of DNase I (ribonuclease-free, electrophoretically purified; Worthington Biochemical Corp.) in 0.01 M sodium acetate (pH 5.1)–2 mM $MnCl_2$. Macaloid, 100 μg/ml, was added to adsorb DNase and was removed by centrifugation; the purified RNA was reprecipitated. One Petri dish yielded 100–150 μg RNA (taking 1 A_{260} Unit to equal 42 μg RNA.)

DNA–RNA hybridization

Highly purified polyoma DNA I, prepared as the 53 S form by sedimentation at pH 12.5 (6), was converted into single strands by boiling for 30 min in 0.1 × SSC [SSC: 0.15 M NaCl–0.015 M Na citrate (pH 7.4)], cooled rapidly by dilution in ice-cold 6 × SSC, and fixed onto 25-mm membrane filters (Schleicher and Schuell, B6) by filtration (7). Filters 3.5 mm in diameter were cut from dried and baked (80°C, 2 hr) filters. A small amount of ^{14}C-labeled polyoma DNA I was added to the unlabeled DNA as a control for the retention (85% or more) and even distribution of the DNA on the filters. Two 3.5-mm filters, each containing 0.2 μg of polyoma DNA, and a blank filter were added to 100 μl of [^{3}H]RNA solution in 4 × SSC in a 300-μl capped polyethylene tube, and incubated at 65°C for 40–48 hr, during which time no appreciable amount of DNA detached from the filters. By studying hybridization kinetics at various RNA concentrations, and the efficiency of hybridization with different amounts of DNA on the filters, we determined these conditions to be optimal for nearly exhaustive hybridization (unpublished data). The filters were washed in 4 × SSC, incubated with 5 μg/ml of pancreatic RNase (treated at 80°C for 15 min to inactivate possible traces of DNase) in 4 × SSC for 1 hr at room temperature, washed extensively in 4 × SSC, dried, and counted. Overlap of ^{14}C into the ^{3}H channel (25%), and of ^{3}H into the ^{14}C channel (0.2%), as well as counting backgrounds, are subtracted from all results.

Abbreviations: Me_2SO, dimethylsulfoxide; [U-^{2}H]Me_2SO, fully deuterated dimethylsulfoxide; SSC, standard saline citrate.

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Polyacrylamide gel electrophoresis

Electrophoresis (8) was performed in a buffer containing 40 mM triethanolamine (pH 7.4), 20 mM sodium acetate, 2 mM EDTA, 2.5% glycerol, and 0.2% sodium dodecyl sulfate (9). 10-cm gels, containing 2.2% acrylamide and 0.11% bis-methylene acrylamide, were polymerized in 0.6-cm (inside diameter) quartz tubes above 1.5-cm cushions of 5% polyacrylamide. Electrophoresis was for 2 hr at 10 V/cm and 22°C. Gels were sliced into 2-mm discs. Half of each slice was solubilized overnight in 0.3 ml of NCS (Amersham-Searle Corp.) plus 10 μl of H_2O, and counted in 5 ml of toluene-based scintillation fluid; the RNA in the other half-slice was eluted with 100 μl of 4 × SSC at 65°C overnight. The eluate was subsequently hybridized in the presence of the gel slice.

Sedimentation in sucrose gradients

RNA samples in 100 μl of 10 mM triethanolamine (pH 7.4)–50 mM NaCl–1 mM EDTA were layered over 4.4-ml, 15–30% (w/w) sucrose gradients in the same buffer, and centrifuged at 54,000 rpm for 1.5 hr at 22°C in a Spinco SW 56 rotor. Fractions were collected from the bottom of the tube, aliquots were dried on filter-paper discs, which were washed at 4°C in 5% trichloroacetic acid and counted, and the remainder of each fraction was adjusted to a concentration of 4 × SSC and hybridized.

Sedimentation in dimethylsulfoxide (Me_2SO)

Gradients of 4.4 ml were formed in polyallomer tubes from equal amounts of the following solutions: (*a*) 89% normal Me_2SO (Merck, Darmstadt), 10% fully deuterated [U-^{2}H]Me_2SO (Centre d'Energie Atomique, Gif-sur-Yvette), 1% H_2O, 1 mM EDTA (pH 7); (*b*) 10% normal Me_2SO, 89% [U-^{2}H]Me_2SO, 1% H_2O, 1 mM EDTA (pH 7) plus 10% ribonuclease-free sucrose (10). RNA samples were dissolved in 5 μl of 1 mM EDTA (pH 7) followed by 100 μl of 99% Me_2SO containing 1 mM EDTA (pH 7), layered onto the gradients, and centrifuged at 48,000 rpm for 6.5 hr at 27°C in a Spinco SW 56 rotor. Aliquots of each fraction were counted on filter papers after washing in 5% trichloroacetic acid, and the fractions were precipitated with 2 volumes of ethanol in the presence of 10 μg of *Escherichia coli* tRNA and 0.2 M NaCl. Pellets were washed in ethanol, resuspended in 100 μl of 4 × SSC, and hybridized.

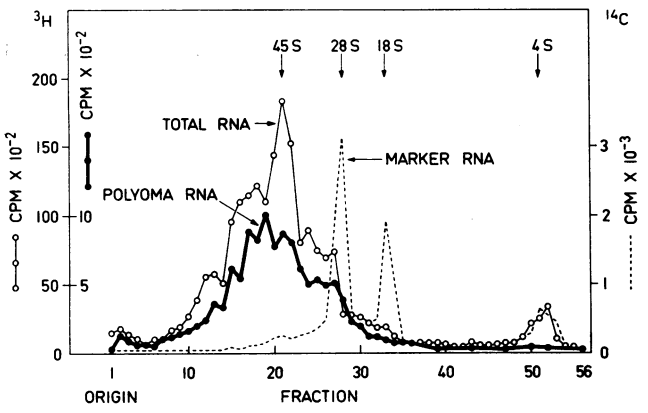

FIG. 1. Electrophoretic analysis of total RNA from polyoma-infected cells labeled for 20 min with [^{3}H]uridine 28 hr after infection. 30 μg of [^{3}H]RNA (13,500 cpm/μg) plus 4.5 μg of RNA from uninfected mouse kidney cells labeled for 27 hr with [^{14}C]uridine ("marker RNA"; 10,900 cpm/μg) were electrophoresed. "Polyoma RNA" (●—●) represents the ^{3}H cpm in ribonuclease-resistant hybrids formed with polyoma DNA by the RNA eluted from half of each 2-mm slice; "total RNA" (O—O) and "marker RNA" (– – –) represent the ^{3}H and ^{14}C cpm, respectively, in the other half-slice. All values are corrected for backgrounds and counting overlap. In this and all subsequent figures, the positions of the 45S, 28S, and 18S ribosomal RNAs and the 4S RNA are marked by *arrows*. Blank filters contained <5 cpm. RNA extracted from uninfected parallel cultures labeled for 20 min shows a similar pattern of total radioactivity, but less than 5 cpm out of 10,000 cpm in the peak fractions hybridized with polyoma DNA.

RESULTS

The size distribution of total RNA from polyoma-infected mouse kidney cells is essentially the same as that from uninfected cells as judged by absorbance and radioactivity patterns in sucrose gradients and gel electropherograms (ref. 2; see below). Virus-specific RNA represents only a fraction of the total RNA synthesized in polyoma-infected cells; therefore, to detect viral RNA species, we have hybridized the RNA from individual gel or gradient fractions with polyoma DNA immobilized on membrane filters (7). Polyoma-specific RNA is defined as the ribonuclease-resistant radioactivity remaining bound to filters after hybridization of radioactive RNA with polyoma DNA.

Hybridizations were performed with excess DNA and for a sufficient time to assure nearly quantitative hybridization (75–90%) of the hybridizable RNA present in each gel or gradient fraction (see *Methods*). Thus, the patterns of polyoma-specific RNA shown in gels or gradients give a good estimate of the actual distribution of hybridizable RNA present.

Size of newly synthesized polyoma-specific RNA

Total RNA was extracted from mouse kidney cultures pulse-labeled for 20 min with [^{3}H]uridine about 28–30 hr after infection. Exhaustive hybridization showed that in 11 different experiments, from 10 to 20% of the total radioactivity incorporated into RNA was in polyoma-specific RNA. RNA from uninfected parallel cultures pulse-labeled, extracted, and hybridized under the same conditions contains less than 0.005% of hybridizable RNA.

Analysis by gel electrophoresis (Fig. 1) shows that the radioactivity incorporated during a 20-min pulse ("total RNA" in Fig. 1) is found mainly in 4–7S RNA, 45S ribosomal precursor RNA, and high molecular weight, polydisperse RNA species present as a broad band between the top of the gel and the 28S ribosomal RNA. Analogous polydisperse RNA has been found in other cells; it is rapidly metabolized nuclear RNA that has been designated "nuclear messenger-like RNA" (11) or "heterogeneous nuclear RNA" (12).

The bulk of the polyoma-specific RNA (Fig. 1) is distributed in a broad peak, which grossly coincides with the nuclear messenger-like RNA. A small proportion (10%) of the polyoma-specific RNA is found between the 28S and 18S ribosomal RNAs, while essentially none of the polyoma-specific RNA migrates more rapidly than 18S ribosomal RNA (0.7×10^6 daltons) (13); this suggests that no extensive breakdown takes place during extraction and analysis of the RNA.

Sedimentation velocity analysis in sucrose gradients (Fig. 2) shows that total radioactivity is present in a broad band extending from about 18 S to >100 S (nuclear messenger-like

RNA), with a superimposed peak of 45S ribosomal precursor RNA and, near the top of the gradient, 4–7S RNA. Total RNA extracted from uninfected parallel cultures, labeled under the same conditions, shows a similar sedimentation pattern.

Polyoma-specific RNA (Fig. 2) exhibits a polydisperse size distribution, similar to that of the total nuclear messenger-like RNA, ranging from about 18 S to greater than 70 S, with the bulk of the RNA sedimenting faster than 28 S.

Estimates of the molecular weight of polyoma-specific RNA, as judged relative to ribosomal RNAs (13), from both electrophoresis and sedimentation experiments, suggest that the bulk of the polyoma-specific RNA is larger than 1.5×10^6 daltons, and that some molecules may be as large as 10×10^6 daltons. This result is surprising, since polyoma DNA, of molecular weight 3×10^6, would be expected to code for RNA with a maximum molecular weight of 1.5×10^6.

The lower size limit of both newly synthesized polyoma-specific RNA and total cellular nuclear messenger-like RNA is smaller as estimated from sedimentation in sucrose gradients than from electrophoretic mobility in gels. Similar differences between apparent molecular weights of nonribosomal RNAs when analyzed by electrophoresis or sedimentation have been noted by others (e.g., 14).

Evidence that "giant" polyoma-specific RNA is not an aggregate of smaller RNA molecules

To exclude the possibility of aggregation, total RNA pulse-labeled for 20 min was incubated in 95% Me_2SO, under conditions known to fully denature double-stranded RNA (ref. 10; see *Methods*). The treated RNA was analyzed directly in sucrose gradients containing 99% Me_2SO as solvent instead of water (Fig. 3). Under these conditions, polyoma-specific RNA remains large and polydisperse, and again exhibits a size distribution closely similar to that of nuclear messenger-like RNA. In this experiment, 60% of the labeled polyoma-specific RNA had an estimated molecular weight (10) greater than 1.5×10^6. We should point out that, relative to ribosomal

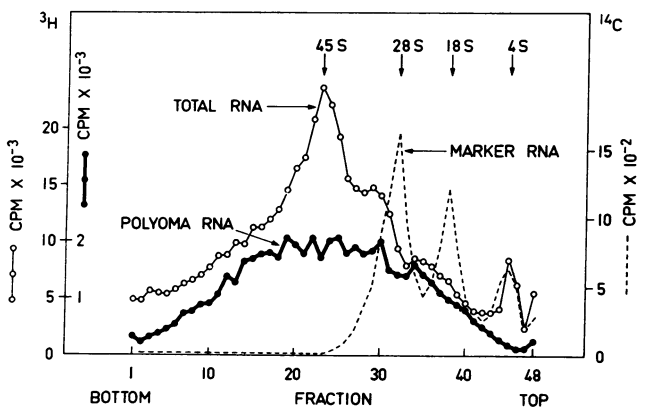

FIG. 2. Sucrose gradient sedimentation analysis of total RNA from polyoma-infected cells labeled for 20 min with [^{3}H]-uridine, 30 hr after infection. 28 μg of [^{3}H]RNA (24,400 cpm/μg), plus 7 μg of ^{14}C marker RNA, were subjected to sucrose gradient analysis. Sedimentation is from *right* to *left*. "Total RNA" (O—O) and "marker RNA" (– – –) represent, respectively, the total ^{3}H and ^{14}C cpm per fraction; "polyoma RNA" (●—●) represents the ^{3}H cpm per fraction hybridized to polyoma DNA.

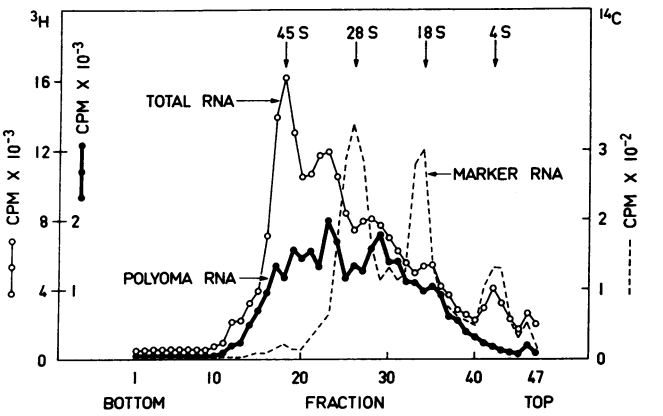

FIG. 3. Me_2SO–sucrose gradient sedimentation analysis of total RNA from polyoma-infected cells labeled for 20 min with [^{3}H]uridine, 28.5 hr after infection. 20 μg of [^{3}H]RNA (20,000 cpm/μg), plus 6 μg of ^{14}C-marker RNA, were subjected to Me_2SO–sucrose gradient sedimentation analysis. Sedimentation is from *right* to *left*.

RNA, total nuclear messenger-like RNA sediments more slowly in Me_2SO gradients than in normal sucrose gradients. The reason for this difference is unknown.

RNA was also analyzed by electrophoresis after treatment with 85% Me_2SO, followed by removal of Me_2SO by reprecipitation (15), or was incubated with 11 M urea and analyzed in the presence of 8 M urea (16). In both cases, the migration of polyoma-specific RNA relative to the ribosomal marker RNAs was essentially unchanged from that of the untreated RNA (Fig. 1).

We therefore conclude that newly synthesized, giant polyoma-specific RNA is not an aggregate of smaller molecules formed during extraction or analysis.

Cleavage of giant polyoma-specific RNA

To study the fate of giant polyoma-specific RNA subsequent to its synthesis, we analyzed RNA samples either pulse-labeled with [^{3}H]uridine for 20 min followed by a 2–4 hr "chase" in unlabeled medium, or labeled continuously for periods of from 2 to 6 hr. The size distributions of polyoma-specific RNA in samples labeled by the two methods are qualitatively similar. Further incorporation of [^{3}H]uridine continues for several hours after the pulse is terminated by replacement of radioactive medium with medium containing unlabeled uridine, as has been reported for other cell systems (17).

Hybridization of total RNA labeled by both methods shows that the amount of polyoma-specific RNA has decreased to 3–6% of the total incorporated radioactivity, as compared with 10–20% in RNA pulse-labeled for 20 min. This suggests that polyoma-specific RNA turns over more rapidly than the major cellular RNA species (ribosomal and transfer RNA).

Analysis by gel electrophoresis of RNA pulse-labeled for 20 min and chased for 2 hr (Fig. 4) shows that much of the total radioactivity is found in the 28S and 18S ribosomal RNAs and in 4–7S species, while the remainder is present in nuclear messenger-like RNA and ribosomal precursor RNAs. RNA extracted from uninfected parallel cultures labeled under the same conditions shows a similar electrophoretic profile, but contains no polyoma-specific RNA.

At least three species of polyoma-specific RNA of lower apparent molecular weight are found in addition to the giant, heterogeneous, polyoma-specific RNA. One peak, which may already be present in the pulse-labeled RNA (Fig. 1), migrates slightly more slowly than 28S ribosomal RNA. A broad band, which in some experiments could be resolved into two distinct peaks, is found between the 18S and 28S ribosomal RNAs.

Sedimentation velocity analysis in sucrose gradients of RNA continuously labeled for 6 hr (Fig. 5) shows that most of the radioactivity is present in 28S and 18S ribosomal RNAs and in 4–7S species, while a small amount of the label is present in nuclear messenger-like RNA and ribosomal precursor RNAs. Hybridization reveals, in addition to giant polyoma-specific RNA, a peak at about 26 S and a broad peak between 16 S and 20 S, which may contain two or more species. These peaks probably correspond to the new peaks seen in electropherograms; however, their apparent molecular weights (determined with respect to ribosomal RNA) are lower as estimated by sedimentation than by electrophoresis.

These results suggest that at least some of the giant polyoma-specific RNA is cleaved after synthesis, leading to the appearance of three (or more) smaller fragments. We cannot exclude the unlikely possibility that the smaller RNAs are synthesized independently of the giant RNA, but at a much slower rate, and are more stable.

DISCUSSION

Our data show that newly synthesized "late" polyoma-specific RNA consists of large, heterogeneous molecules, the bulk of which ("giant RNA") have molecular weights greater than 1.5×10^6, the size expected for a single transcript of the polyoma genome. Treatment with Me_2SO or urea has shown that this giant RNA is not an aggregate of smaller RNA molecules. Giant polyoma-specific RNA shows a striking similarity in size distribution to nuclear messenger-like RNA (heterogeneous nuclear RNA) of the host cell. Some virus-specific RNA larger than the viral genome has been reported to occur (18, 19) in cells lytically infected with simian virus 40 (SV40), which is similar to polyoma virus.

Subsequent to its synthesis, at least some of the giant polyoma-specific RNA apparently undergoes cleavage to three or more smaller species. Some of the giant RNA may also be rapidly degraded. Definite assignment of molecular weights to the smaller RNA species is precluded by their different behavior in sucrose gradients and gels. However, the species with a sedimentation coefficient of about 26 S in sucrose gradients may correspond to the complete transcript of a strand of polyoma DNA (1.5×10^6 daltons), while the 16–20S species may be cleavage products of the 26S RNA.

To explain the paradox that much of the newly synthesized polyoma-specific RNA is larger than the polyoma genome, we may consider two of the most likely models of polyoma transcription:

Continuous Transcription. Polyoma DNA being circular, transcription could continue for several rounds rather than being terminated after one cycle.

Transcription from "Integrated" DNA. Transcription may be restricted to polyoma DNA molecules that are covalently attached ("integrated") to host cell DNA (e.g., if polyoma DNA lacked promoter sites for the transciption of late RNA). Adjacent host cell genes might then be transcribed along with viral genes, giving rise to RNA molecules containing both host and viral genetic information. Certain models of this type could explain the similarity in size distribution of newly synthesized polyoma-specific RNA and nuclear messenger-like RNA of the host cell.

Recent results (unpublished) suggest that "early" polyoma-specific RNA (transcribed in the absence of detectable viral DNA synthesis) is also synthesized as giant molecules. This points to the possibility that the expression of the viral genome may be controlled at the level of processing (selective cleavage and/or degradation) of giant virus-specific RNA, rather than at the level of transcription of "early" and "late" genes 20–22).

Certain features of polyoma-infected cells make this system potentially useful for the study of messenger RNA. In most eukaryotic cell systems, no well-defined messenger RNA is produced in large amounts, and no direct means of locating a given RNA species from within the mass of cellular RNA is available. In the polyoma–mouse kidney cell system, virus-specific RNA is synthesized in relatively large amounts and can be detected by DNA–RNA hybridization as soon as it is

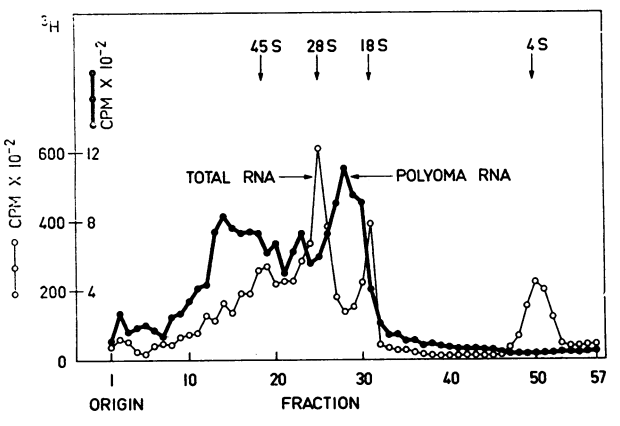

FIG. 4. Electrophoretic analysis of total RNA from polyoma-infected cells labeled for 20 min with [^{3}H]uridine, 28.5 hr after infection, and subsequently incubated for 2 hr in nonradioactive medium containing 10 μg/ml of uridine. 20 μg of [^{3}H]RNA (41,700 cpm/μg) was electrophoresed.

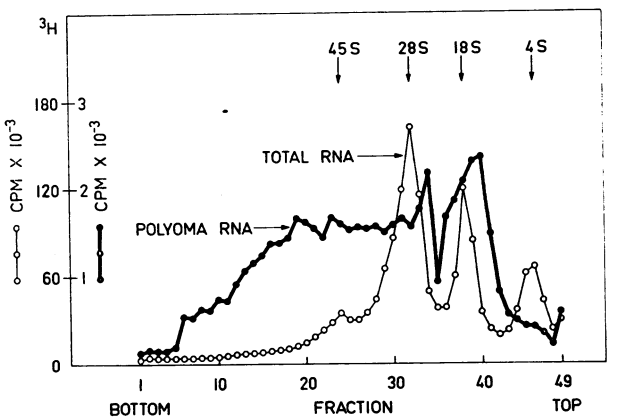

FIG. 5. Sucrose gradient sedimentation analysis of total RNA from polyoma-infected cells labeled with [^{3}H]uridine for 6 hr. 26 μg of [^{3}H]RNA (94,000 cpm/μg) isolated from cells labeled from 24.5 to 30.5 hr after infection was analyzed on a sucrose gradient.

made in the nucleus. Thus, it should be possible to study the synthesis, packaging, metabolism, and transport of specific RNA molecules destined to serve as messengers for the production of viral proteins.

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1. Benjamin, T. L., *J. Mol. Biol.*, **16**, 359 (1966).
2. Hudson, J., D. Goldstein, and R. Weil, *Proc. Nat. Acad. Sci. USA*, **65**, 226 (1970).
3. Winocour, E., *Virology*, **19**, 158 (1963).
4. Pétursson, G., and R. Weil, *Arch. Gesamte Virusforsch.*, **24**, 1 (1968).
5. Scherrer, K., in *Fundamental Techniques in Virology*, ed. K. Habel and N. P. Salzman (Academic Press, New York, 1969), pp. 413–432.
6. Hudson, J. B., *Eur. J. Biochem.*, **9**, 112 (1969).
7. Gillespie, D., and S. Spiegelman, *J. Mol. Biol.*, **12**, 829 (1965).
8. Loening, U. E., *Biochem. J.*, **102**, 251 (1967).
9. Mirault, M.-E., and K. Scherrer, submitted to *Eur. J. Biochem.*
10. Strauss, J. H., Jr., R. B. Kelly, and R. L. Sinsheimer, *Biopolymers*, **6**, 793 (1968).
11. Scherrer, K., L. Marcaud, F. Zajdela, I. M. London, and F. Gros, *Proc. Nat. Acad. Sci. USA*, **56**, 1571 (1966); Scherrer, K., and L. Marcaud, *J. Cell. Physiol.*, **72**, Suppl. 1, 181 (1968).
12. Soeiro, R., H. C. Birnboim, and J. E. Darnell, *J. Mol. Biol.*, **19**, 362 (1966).
13. McConkey, E. H., and J. W. Hopkins, *J. Mol. Biol.*, **39**, 545 (1969).
14. Attardi, G., Y. Aloni, B. Attardi, D. Ojala, L. Pica-Mattoccia, D. L. Robertson, and B. Storrie, *Cold Spring Harbor Symp. Quant. Biol.*, **35**, 599 (1970).
15. Katz, L., and S. Penman, *Biochem. Biophys. Res. Commun.*, **23**, 557 (1966).
16. Dobos, P., and P. Faulkner, *J. Virol.*, **6**, 145 (1970).
17. Warner, J. R., R. Soeiro, H. C. Birnboim, M. Girard, and J. E. Darnell, *J. Mol. Biol.*, **19**, 349 (1966).
18. Martin, M. A., and J. C. Byrne, *J. Virol.*, **6**, 463 (1970).
19. Tonegawa, S., G. Walter, A. Bernardini, and R. Dulbecco, *Cold Spring Harbor Symp. Quant. Biol.*, **35**, 823 (1970).
20. Aloni, Y., E. Winocour, and L. Sachs, *J. Mol. Biol.*, **31**, 415 (1968).
21. Oda, K., and R. Dulbecco, *Proc. Nat. Acad. Sci. USA*, **60**, 525 (1968).
22. Carp, R. I., G. Sauer, and F. Sokol, *Virology*, **37**, 214 (1969).