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Adenovirus Messenger RNA in Mammalian Cells: Failure of Polyribosome Association in the Absence of Nuclear Cleavage

(toyocamycin/HeLa cells/viral transcripts)

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ABSTRACT **The nuclear synthesis of adenovirus-specific RNA late in the infectious cycle in the presence of toyocamycin (an adenosine analogue) has been investigated. There is reduced synthesis of viral RNA with an accumulation of virus-specific RNA in the molecular weight range of at least 4 to 8 × 10^6. No new viral RNA associates with cytoplasmic polyribosomes. In addition, hybridization competition experiments indicate a 70% competition between these large nuclear transcripts and polyribosome-associated viral RNA that was synthesized in the absence of inhibitor. These data are consistent with the following interpretations: complete nuclear processing of viral RNA is necessary for polyribosome association, and precursor viral message(s) contain sequences that are lost normally during post-transcriptional processing.**

Evidence has been presented that messneger RNA(mRNA) in mammalian cells may undergo a process of cleavage after transcription. A precursor–product relationship has been suggested between nuclear heterogeneous RNA of high molecular weight and the mRNA of lower molecular weight that is associated with polysomes in HeLa cells (1). In addition, cells either lytically infected or transformed by DNA nuclear viruses contain larger virus-specific RNA transcripts in the nucleus than in the cytoplasm (2, 3). To examine in more detail the nature of this cleavage of mRNA and its possible implications in mammalian cells, it would be helpful if the processes of synthesis and cleavage could be separated. This separation has been achieved by the use of toyocamycin in studies on processing of ribosomal RNA (4). When used in relatively low concentrations, this inhibitor is incorporated into the newly synthesized 45S RNA resulting in the subsequent failure of processing. In the present study, the effect of toyocamycin on processing of viral RNA has been investigated. HeLa cells, at 22 hr after infection with adenovirus 2, were incubated with this inhibitor and the synthesis, accumulation, and fate of nuclear viral transcripts were ascertained. The data demonstrate that in the presence of inhibitor large virus-specific transcripts are synthesized and accumulate in the nucleus, but fail to be processed. Under these conditions, no virus-specific RNA becomes associated with cytoplasmic polyribosomes.

MATERIALS AND METHODS

Maintenance of Cells. HeLa S3 cells were maintained in suspension culture in Eagle's medium (5) supplemented with 7% calf serum.

Preparation of Virus and Infection of Cells. Stocks of adenovirus type 2 were prepared and stored as described by Maizel *et al.* (6). Virus preparations were used within 1 month and were free of identifiable, small, adeno-associated virus. Cells were infected at a concentration of 2000 viral particles per cell as described (7).

Preparation of Nuclear RNA. Nucleoplasm was prepared from HeLa cells by the procedure of Penman (8). Nucleic acid and protein were precipitated at −20° for 16 hr with 0.15 M sodium chloride, 0.1% of 2-mercaptoethanol, and 2.5 volumes of ethanol. This precipitate was suspended in 2.0 ml STE [0.05 M Tris·HCl (pH 7.4) with 1 mM EDTA and 0.1 M NaCl] containing 1% sodium dodecyl sulfate (SDS) and extracted three times with a phenol solution composed of 80% phenol and 20% STE. RNA was precipitated at −20° for 16 hr from the aqueous layer as above.

Preparation of Polyribosome-Associated RNA. Cytoplasmic polyribosomes were prepared with Nonidet P-40 followed by separation in linear 7.5–45% sucrose gradients as described (9). An alternative method, centrifugation through 50% sucrose in RSB [0.01 M NaCl, 1.5 mM $MgCl_2$, 0.01 M Tris·HCl (pH 7.2)] buffer at 60,000 rpm (in an SW65 rotor) for 2.5 hr at 5°, was also used (2). RNA was released from the polyribosome fraction by incubation at 37° for 30 min with 1% SDS, 1 mM EDTA, and 0.1% of 2-mercaptoethanol. RNA was concentrated and extracted with phenol as above.

Sedimentation Analysis of RNA (SDS–Sucrose Gradients and Dimethylsulfoxide Gradients). Samples of RNA to be analyzed in SDS–sucrose gradients were solubilized in 0.5 ml of PEMS [0.01 M sodium phosphate buffer (pH 7.4) containing 1% SDS, 0.1% of 2-mercaptoethanol, 0.1 M NaCl, and 0.02 M EDTA]. RNA was sedimented in 16 ml 15–30% (w/w) linear sucrose gradients consisting of 0.01 M Tris·HCl (pH 7.2), 0.5% SDS, and 0.1 M NaCl. Absorbance at 260 nm was monitored, 0.75-ml fractions were collected, and the RNA in each fraction was precipitated in the presence of 17.6 *A* units of yeast transfer RNA. S values were calculated according to the method of Martin and Ames (10) with 28S and 18S ribosomal RNA as markers of sedimentation. Precipitates were collected and resuspended in 2.0 ml of 2-fold SSC (0.15 M sodium chloride and 0.015 M sodium citrate). Total Cl_3CCOOH-insoluble radioactivity and virus-specific RNA were determined as described below.

Abbreviations: SDS, sodium dodecyl sulfate; Me_2SO, dimethylsulfoxide; SSC, 0.15 M NaCl–0.015 M sodium citrate.

Sedimentation in 5 ml of 99% dimethylsulfoxide (Me_2SO) gradients was done by the procedure of Strauss *et al.* (11). 20- to 25-μg Samples of RNA in 0.01 ml of 0.01 M NaCl were diluted with 0.09 ml of Me_2SO and 0.01 ml of dimethylformamide and layered on the above gradients, which were centrifuged in an SW65 rotor for 15 hr at 50,000 rpm at 27°. Seven-drop fractions were collected from the bottom of the gradient with a probe and a Buchler pump. The RNA in each fraction was precipitated as above and resuspended in 2.0 ml of 2-fold SSC for the determination of virus-specific RNA. Molecular weight calibration of the gradient was determined from the rate of sedimentation of isolated cellular 45S, 28S, and 18S marker RNA.

DNA–RNA Hybridization and Hybridization Competition. Annealing between RNA and alkaline-denatured viral DNA was performed essentially by the procedure of Gillespie and Spiegelman (12). Adenovirus DNA was prepared from purified virions by the method of Levine and Ginsberg (13).

Determination of RNA. RNA was quantified by the method described by Shatkin (14). Nuclear preparations of 2 to 4 $\times$ 10^7 cells routinely contained 20–50 μg of RNA.

Reagents. [5-^{3}H]uridine (20 Ci/mmol) was obtained from New England Nuclear Corp., Boston, Mass. Dimethyl-d_6-sulfoxide was obtained from International Chemical and Nuclear Corp., Irvine, Calif. Toyocamycin (4-Amino-5-cyano-7-β-D-ribofuranosyl-pyrrolo[2,3-*d*]pyrimidine) was a gift from George Acs.

RESULTS

Reduced synthesis of viral RNA

When nuclear adenovirus-specific RNA is examined, the amount present is determined by the relative rates of synthesis of viral transcripts, their post-transcriptional processing, and their transport into the cytoplasm. At 22 hr after infection, when 80–100% of the adenovirus genome is expressed (3), this synthesis and accumulation of RNA in the nucleus was examined in the presence and absence of inhibitor. Cells were concentrated and exposed to radioactive uridine, and nuclear RNA was prepared and analyzed to determine the rate of synthesis of total RNA and adenovirus-specific RNA. In the absence of inhibitor, the amount of newly synthesized RNA was maximal by 30 min (Fig. 1*A*). In contrast, maximal amounts of RNA in cells treated with inhibitor were not detected until 60–90 min and represented 25% of the acid-precipitable radioactivity of untreated cells (Fig. 1*A*). In the first 15 min about 92% of the maximum was noted in untreated cells, whereas 40% of the maximum was observed in the treated cells.

Without toyocamycin, the amount of adenovirus-specific RNA was maximal by 30 min, and the pattern of accumulation was similar to that of total nuclear RNA (Fig. 1*B*). However, the synthesis and accumulation of virus-specific RNA was at a slower rate and did not reach a maximum in the presence of toyocamycin, even by 120 min. At this time the amount of virus-specific RNA is 25% of that found in control cells. About 7–8% of the total radioactivity in both treated and untreated cells was hybridizable. The maximum noted in the accumulation of adenovirus-specific RNA at 30 min in untreated cells could result from limiting amounts of DNA per filter or exhaustion of radioactive uridine. The former explanation seems unlikely because of the similarity between the kinetics of accumulation of total RNA (Fig. 1*A*) and hybridizable RNA (Fig. 1*B*). To obtain additional evidence that the DNA content per filter was not limiting, aliquots of the 90-min sample containing 20, 60, and 140% of the amount originally present in the hybridization mixture were annealed to filters containing 10 μg of DNA. Since the quantity of RNA annealed was directly proportional to the amount of RNA exposed (Fig. 1*B*, *insert*), the DNA content per filter appears not to be limiting. Exhaustion of radioactive uridine also seems unlikely since the results of experiments with 25 μCi/ml, 100 μCi/ml, or 200 μCi/ml were essentially as described above. Increasing the dose of toyocamycin resulted in about 80% inhibition of viral RNA synthesis, which became asymptotic at 8.75 μg/1 $\times$ 10^7 cells.

Evidence for the accumulation of large nuclear viral transcripts

For determination of the nature of the virus-specific RNA synthesized in the presence of inhibitor, nuclear RNA was analyzed by sedimentation in SDS–sucrose and Me_2SO gradients. Nuclear RNA was prepared from control cells exposed to pulse labeling conditions of 10 min and equilibrium labeling conditions of 60 min. Virus-specific RNA labeled for short periods of time sedimented throughout the SDS–sucrose gradient with major peaks appearing at the 52S, 36S, and 24S regions (Fig. 2*A*). Of the total virus-specific RNA in the gradient, about 39% sedimented faster than 45 S and 15%

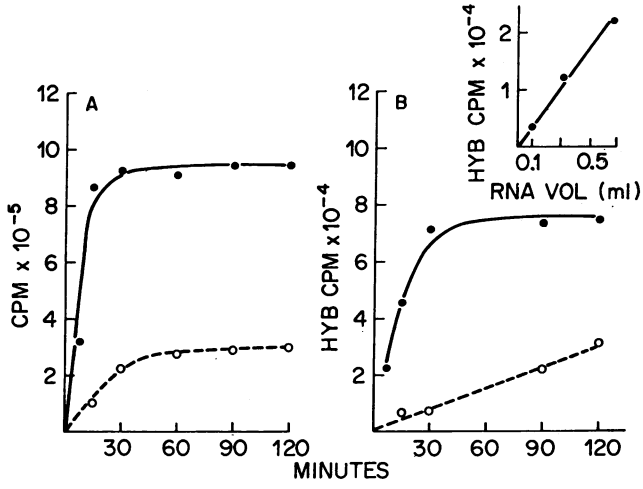

FIG. 1. Kinetics of nuclear RNA synthesis in the presence of toyocamycin. At 22 hr after infection, cells were incubated for 15 min with 1.75 μg of toyocamycin/1 $\times$ 10^7 cells and suspended at a concentration of 1 $\times$ 10^7/ml in complete warmed medium at 34° containing 50 μCi/ml of [^{3}H]uridine and 1.75 μg/ml of inhibitor. At the indicated times, 2.0-ml aliquots of each culture were removed and chilled, and nuclear RNA was prepared and precipitated (see *Methods*). RNA was resuspended in 2.0 ml of 2-fold SSC, and aliquots were used to determine total Cl_3CCOOH-insoluble radioactivity and virus-specific RNA with filters containing about 10 μg of denatured adenovirus DNA. The data have been adjusted to total radioactivity present in the sample. (*B*, *Insert*) Hybridization was performed as described above with aliquots of 0.1, 0.3, and 0.7 ml of the 90-min sample of the control culture. (*A*) Total radioactivity (●——●) without inhibitor; (O- - -O) with inhibitor. (*B*) Hybridizable radioactivity (●——●) without inhibitor; (O- - -O) with inhibitor.

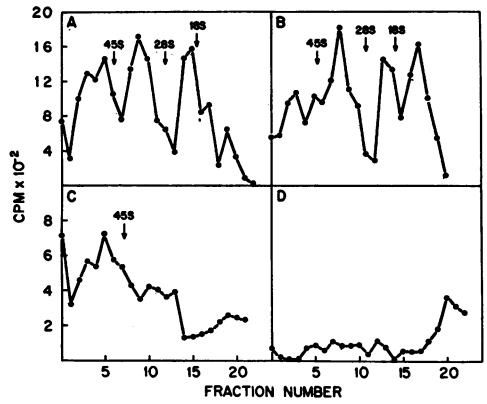

FIG. 2. Sedimentation analysis of nuclear adenovirus-specific RNA in SDS–sucrose gradients. Radioactive nuclear RNA was prepared from 2×10^7 cells, processed as described in Fig. 1, and separated in 15–30% SDS–sucrose gradients (see *Methods*) by centrifugation in an SW27 rotor at 20,000 rpm for 16 hr at 20°. Virus-specific RNA in each fraction was determined with nitrocellulose filters containing about 3 μg of denatured viral DNA. The rate of migration of cellular markers, 45S, 28S, and 18S RNA, was determined in parallel gradients. (*A*) Profile of hybridizable RNA after 10-min labeling time. (*B*) Profile of hybridizable RNA after 60-min labeling time. (*C*) Profile of hybridizable RNA after 60-min labeling time in the presence of toyocamycin at a concentration of 1.75 $\mu g/1 \times 10^7$ cells. (*D*) Profile of hybridizable RNA after 60-min labeling time in the presence of toyocamycin at a concentration of 17.5 $\mu g/1 \times 10^7$ cells.

sedimented slower than 18 S. A similar profile of sedimentation was observed for viral RNA labeled for 60 min, with 29% sedimenting greater than 45 S and 27% sedimenting less than 18 S (Fig. 2*B*). However, in cultures treated with inhibitor that were exposed to label for 60 min, about 50% of the total RNA sedimented in regions of the gradient greater than 45 S. Major peaks of sedimentation at 36 S, 24 S, and 10 S were not observed (Fig. 2*C*). In the presence of 10-fold excess of toyocamycin, little virus-specific RNA was detected (Fig. 2*D*), confirming the dosage effects previously described.

To eliminate the possibilities that these results were due to aggregation of RNA or persistent DNA–RNA hybrids and to estimate the range of molecular weight of virus-specific RNA, about 5–10 μg of nuclear RNA from cells treated with inhibitor and from untreated cells was separated in 99% Me_2SO gradients (Fig. 3). Species of cellular RNA were used as markers of sedimentation. The molecular weights of 45S, 28S, and 18S RNA reported in the literature as 4.4×10^6, 1.9×10^6, and 0.6×10^6, respectively (15), were used to calibrate the gradients (Fig. 3*B*). Virus-specific RNA synthesized in the presence of inhibitor sedimented predominantly in the region of the gradient corresponding to a range of molecular weight of 4 to 8×10^6. Consistent with the sedimentation pattern in SDS gradients, three major peaks of viral RNA were observed from nuclear preparations of untreated cells. These sedimented in regions of the gradient corresponding to molecular weight ranges of 4 to 8×10^6, 1.8 to 2.0×10^6, and 0.8 to 1.0×10^6, respectively. In addition, the more slowly sedimenting polydispersed RNA in the less than 0.6×10^6 molecular weight range, noted in the inhibitor-treated cultures, was greatly reduced.

Failure of polyribosome association

The previous data indicate that synthesis of nuclear viral transcripts in the presence of inhibitor results in the accumulation of viral RNA in a molecular weight range of at least 4 to 8 $\times 10^6$. Viral RNA of this size has not been shown to be associated with polyribosomes (3). To determine if these large transcripts would exit from the nucleus and function as mRNA, polyribosome-associated RNA synthesized in the presence and absence of inhibitor was analyzed. Cells were pulsed for 60 min, cytoplasm was prepared, and polyribosomes were separated in 7.5–45% sucrose gradients. Initial experiments with treated and untreated cells demonstrated that the peak of sedimentation of the polyribosomes was at about 160 S. Thus, no increase in the rate of sedimentation was noted in cells treated with toyocamycin as would have been expected if large, unprocessed viral transcripts had associated with polyribosomes. In the absence of inhibitor, virus-specific RNA did sediment with cellular polyribosomes. About 70% of this hybridizable virus-specific RNA was associated with polyribosomes, since it no longer sedimented as heavy material after the polyribosomes had been disrupted by EDTA (16, 17). This virus-specific RNA extracted from the greater than 100S region of the gradient sedimented with a major peak at 26 S and with shoulders at 36 S and 22 S (Fig. 4*A*). About 28–30% of the radioactive RNA associated with polyribosomes hybridized. In the treated cells, the amount of radioactivity sedimenting beyond the 100S region was reduced about 33%. However, this RNA, when extracted and analyzed in SDS–sucrose gradients, revealed insignificant amounts of virus-specific RNA (Fig. 4*B*).

Reduced hybridization competition between large nuclear transcripts and polyribosome-associated transcripts

Analysis of nuclear and polyribosome-associated viral RNA in SDS–sucrose gradients demonstrated that, unlike the polyribosome-associated RNA (Fig. 4*A*), a fraction of the virus-specific nuclear transcripts exhibits a sedimentation velocity

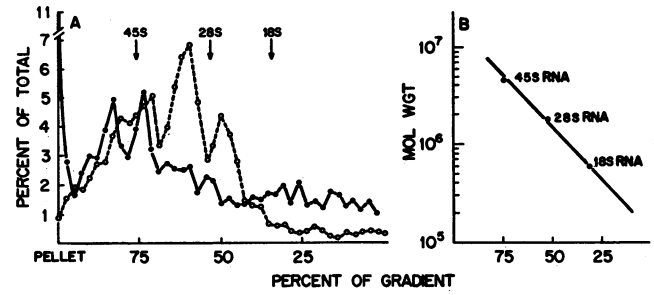

FIG. 3. Range of molecular weights of adenovirus-specific nuclear RNA synthesized in the presence of toyocamycin. (*A*) Radioactive nuclear RNA was prepared from 2×10^7 cells that had been exposed to [^{3}H]uridine at 50 μCi/ml for 10 and 60 min. The latter incubation mixture also contained toyocamycin at a concentration of 1.75 $\mu g/1 \times 10^7$ cells. About 25% of the RNA of each preparation was separated in Me_2SO gradients (see *Methods*). The distribution of viral RNA was determined with filters containing about 3 μg of denatured adenovirus DNA. For convenience, the data are presented as percent of total radioactivity and are plotted on a single graph. Viral RNA synthesized in the presence of toyocamycin (●——●). Viral RNA synthesized in the absence of toyocamycin (O- - -O). (*B*) Calibration of the Me_2SO gradient with markers 45S, 28S, and 18S RNA.

greater than 35 S (Fig. 2*A*). Cosedimentation analyses on a single sucrose gradient of dual labeled nuclear and polyribosome-associated viral RNA confirmed this observation. Recently, it has been demonstrated that the lower molecular weight cytoplasmic and polyribosome-associated RNA contain all the sequences of the nuclear virus-specific RNA sedimenting faster than 35 S (3). We have examined this question with regard to RNA synthesized in the presence of toyocamycin, particularly since under these conditions sedimentation analysis has revealed that the percent of viral RNA pelleted in both Me_2SO– and SDS–sucrose gradients is increased 5- to 10-times. To ascertain whether this nuclear viral RNA with a molecular weight greater than or equal to 4 to 8 $\times$ 10^6, synthesized in the presence of toyocamycin, would hybridize satisfactorily and to determine the degree of competition with polyribosome-associated RNA, a competitive hybridization experiment was performed (Fig. 5). The viral RNA of high molecular weight synthesized in the presence of inhibitor did saturate filters containing about 0.1 μg of denatured viral DNA (Fig. 5, *insert*). Previous incubation with increasing amounts of polyribosome-associated RNA synthesized in the absence of inhibitor results in a competition of about 70%. As a control, competition between radioactive and nonradioactive RNA sedimenting greater than 35 S, both synthesized in the presence of inhibitor, resulted in at least 95% competition.

DISCUSSION

Synthesis of nuclear adenovirus-specific RNA in the presence and absence of toyocamycin and its subsequent association with cytoplasmic polyribosomes have been investigated. It appears that nuclear RNA synthesized in the presence of this inhibitor is not processed, or is at best only partially processed This conclusion is derived from a sedimentation analysis of virus-specific RNA. 60% of this RNA sedimented with a velocity equal to or greater than 45 S in SDS–sucrose gradients,

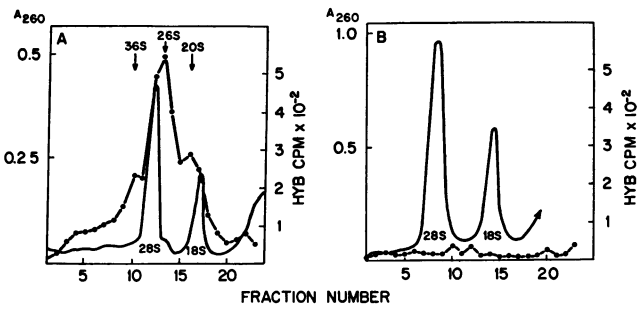

FIG. 4. Sedimentation analysis of polyribosome-associated virus-specific RNA in cells treated with toyocamycin. At 22 hr after infection, 12 $\times$ 10^7 cells were treated with toyocamycin and suspended in complete warmed medium at 34° containing inhibitor and radioactive uridine as described in the legend of Fig. 1. 6 $\times$ 10^7 Control cells were similarly treated but without inhibitor. After incubation at 34° for 1 hr, cells were processed for polyribosomes. Polyribosomes sedimenting greater than 100 S were pooled, and RNA was prepared. This RNA was separated in 15–30% SDS–sucrose gradients in an SW27 rotor at 23,000 rpm for 20 hr at 20°. Virus-specific RNA was determined as described in Fig. 2. (*A*) Pattern of virus-specific RNA synthesized in the absence of toyocamycin. (*B*) Pattern of virus-specific RNA synthesized in the presence of toyocamycin. Absorbance at 260 nm (——); virus-specific RNA (●——●).

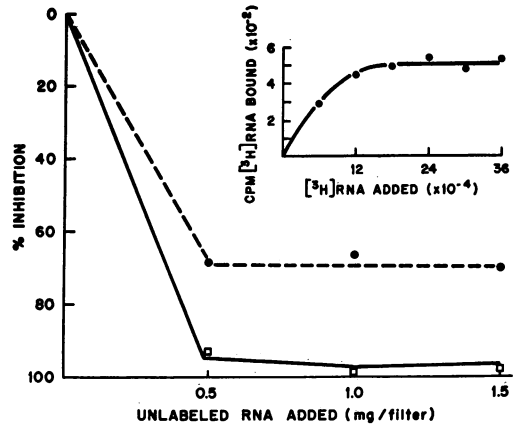

FIG. 5. Hybridization competition of nuclear RNA of high molecular weight synthesized in the presence of toyocamycin. At 22 hr after infection, cells were incubated for 1 hr with and without [^{3}H]uridine in the presence of toyocamycin, and nuclear RNA was prepared as described in the legend of Fig. 1. RNA in both preparations sedimenting greater than 35 S was pooled with pellet fractions and was concentrated by ethanol precipitation. Polyribosome-associated RNA synthesized in the absence of inhibitor and [^{3}H]uridine was prepared (3). Filters containing about 0.1 μg of denatured adenovirus DNA were exposed to increasing amounts of unlabeled, greater than 35S RNA, synthesized in the presence of toyocamycin (□– – –□); and polyribosome-associated RNA (●– – –●) synthesized in the absence of inhibitor. After 17 hr of incubation at 66°, the filters were washed and exposed to saturating amounts of radioactive nuclear RNA, i.e., 36 $\times$ 10^4 cpm (*insert*). The filters were washed, and residual RNase-resistant radioactivity was ascertained.

corresponding to a minimum molecular weight of 4 to 8 $\times$ 10^6. In contrast, nuclear viral RNA synthesized in the absence of inhibitor sedimented throughout the gradient with prominent peaks at 52 S, 36 S, and 26 S, corresponding to approximate molecular weights of 4 $\times$ 10^6, 2.2 $\times$ 10^6, and 1.2 $\times$ 10^6, respectively. These data argue for the accumulation of initial viral transcripts and the subsequent failure of cleavage. This newly synthesized virus-specific RNA is absent from cytoplasmic polyribosomes. This suggests that cleavage of viral mRNA is essential for its eventual translation in the cytoplasm.

Sedimentation analysis consistently demonstrated an increase of 5- to 10-fold in the percent of pelleted viral RNA from cells treated with inhibitor over that from untreated cells. These results suggest that initial unprocessed viral transcripts have accumulated that are even larger than those that have been displayed previously by analyses in acrylamide gels (3). Thus, viral RNA synthesized in the presence of toyocamycin could represent transcription products coded for by considerably more than 50% of the viral genome. Because of this large size, velocity gradients have provided the simplest method for their isolation; analysis by gel electrophoresis has not been satisfactory. The heterogeneity of viral RNA sedimenting faster than 45 S could represent inadequate separation of transcribed products, a series of partially transcribed products, or fully transcribed products with partial cleavage to intermediates. Nevertheless, RNA synthesized in the presence of inhibitor did hybridize satisfactorily with denatured adenovirus DNA, and apparently possessed more virus-specific RNA nucleotide sequences than virus-specific RNA that as-

sociated with polyribosomes in the absence of inhibitor. Improper processing may, therefore, lead to the accumulation of large, nuclear mRNA precursors that retain RNA sequences that are lost normally. It is possible that the large nuclear products are not as apparent when synthesized in the absence of toyocamycin because cleavage normally occurs during the process of synthesis before the completion of transcription. Preliminary experiments with radioactive toyocamycin indicate that the nucleotide analogue is incorporated into these large nuclear transcripts.

Although it seems unlikely, it is conceivable that the nucleus contains two populations of viral RNA. One population could be very large and turn over like nuclear heterogeneous RNA. A second population of smaller size could be independently synthesized and act as viral messenger. This would imply that the large nuclear viral RNA is not a precursor to the smaller polyribosome-associated viral RNA. However, pulse-chase experiments in our laboratory with actinomycin D have indicated that viral RNA sedimenting greater than 45 S is precursor to nuclear RNA sedimenting less than 45 S. These results are consistent with the precursor-product relationship previously suggested from competitive hybridization experiments (3).

The failure of large viral transcripts to appear in the polyribosomes provokes speculation concerning the nature and necessity of the cleavage process. The failure of ribosomal precursor RNA (45 S) and viral transcripts to be processed is consistent with the idea that viral and host cleavage enzymes are identical. It is also conceivable that the enzymatic activities are different and that the inhibition of cleavage is the result of an altered primary sequence or secondary folding in the RNA substrate. In the light of hypotheses concerning the significance of polyadenylic acid sequences in mRNA of mammalian cells (18), it is interesting that our results have been obtained with an analogue of adenosine. Both nuclear and polyribosome-associated viral RNA contain polyadenylic acid sequences that may be coded for by the host cell (18). Post-transcriptional cleavage may be necessary for the attachment of these sequences. For example, there is evidence that the polyadenylic sequences are attached to the 3′-end of *in vitro* mRNA synthesized by vaccinia viral cores (19). As suggested by others, these sequences may be involved in the transport of mRNA from the nucleus or formation of polyribosomes once mRNA is in the cytoplasm (18, 20). For example, processed viral transcripts may combine with transport proteins at the polyadenylic site. Unprocessed viral transcripts lacking polyadenylic sequences, thus, would be incapable of transport. Finally, it would be of interest to determine whether initial viral transcripts, as well as polyribosome-associated mRNA, would initiate protein synthesis in a cell-free preparation. It is conceivable that uncleaved viral RNA will not function in either mammalian or *Escherichia coli* protein synthesis systems, whereas specifically cleaved products will be active.

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