TRANSPORT OF VIRAL RNA IN KB CELLS INFECTED WITH ADENOVIRUS TYPE 2

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ABSTRACT

Messenger RNA transport was studied in KB cells infected with the nuclear DNA virus adenovirus type 2. Addition of 0.04 μg/ml of actinomycin completes the inhibition of ribosome synthesis normally observed late after infection and apparently does not alter the pattern of viral RNA synthesis: Hybridization-inhibition experiments indicate that similar viral RNA sequences are transcribed in cells treated or untreated with actinomycin. The polysomal RNA synthesized during a 2 hr labeling period in the presence of actinomycin is at least 60% viral specific. Viral messenger RNA transport can occur in the absence of ribosome synthesis. When uridine-^{3}H is added to a late-infected culture pretreated with actinomycin, viral RNA appears in the cytoplasm at 10 min, but the polysomes do not receive viral RNA-^{3}H until 30 min have elapsed. Only 25% of the cytoplasmic viral RNA is in polyribosomes even when infected cells have been labeled for 150 min. The nonpolysomal viral RNA in cytoplasmic extracts sediments as a broad distribution from 10S to 80S and does not include a peak cosedimenting with 45S ribosome subunits. The newly formed messenger RNA that is ribosome associated is not equally distributed among the ribosomes; by comparison to polyribosomes, 74S ribosomes are deficient at least fivefold in receipt of new messenger RNA molecules.

INTRODUCTION

KB cells infected with adenovirus type 2 (Ad 2)[1] provide a model system for the study of RNA transport. In a process presumably analogous to messenger RNA (mRNA) transport in uninfected cells, viral RNA molecules are transcribed from nuclear viral DNA and transported to the cytoplasm for translation (Thomas and Green, 1966; Velicer and Ginsberg, 1968). The advantages of using the Ad 2 system for these experiments are threefold: Late after infection (18 hr), ribosome synthesis is reduced at least 80% (Raskas, Thomas, and Green, 1970). Second, the population of viral RNA molecules is far less heterogeneous than that of cell messenger RNA. Since late after infection 80–100% of the 23 × 10^6 daltons of the genome is transcribed (Thomas and Green, 1969; Fujinaga and Green, 1970), there might be 25 viral mRNA cistrons coding for proteins of average molecular weight 50,000. Recent experiments have documented the presence of approximately 10 molecular viral RNA species in late-infected cells (Parsons, Gardner, and Green, in preparation). An additional advantage of the Ad 2 system is the ability to hybridize viral RNA to viral DNA without the ambiguities involved in

[1] *Abbreviations used in this article:* Ad 2, adenovirus type 2; HEPES, *N*-2-hydroxyethylpiperazine-N′-2-ethane sulfonic acid; mRNA, messenger RNA; PFU, plaque-forming units; SDS, sodium dodecyl sulfate; SSC, standard saline citrate.

annealing cell RNA to cell DNA (Soeiro and Darnell, 1970; Britten and Kohne, 1968; McCarthy, 1968; Melli and Bishop, 1969).

In this report we describe the kinetics of transport and cytoplasmic distribution of viral RNA late after infection with Ad 2.

METHODS

Cells, Viral Infection, and Preparation of Cytoplasmic Extracts

Exponentially growing KB cell cultures were concentrated to a density of approximately 1.25×10^7 cells per milliliter and infected with Ad 2 virus at an input multiplicity of 30–50 plaque-forming units (PFU) per cell. After 1 hr to permit viral adsorption, infected cells were resuspended at densities of 225,000–300,000 cells per milliliter. Cytoplasmic extracts were prepared from cells in hypotonic medium by treating with nonidet P40 (Borun, Scharff, and Robbins, 1967) as described previously (Raskas Thomas, and Green, 1970).

Labeling Procedures

Cellular RNA was often uniformly labeled with uridine-^{14}C (0.25 μCi/ml; 57 mCi/mmole) for 24 hr before viral infection (as in Fig. 6). To label new RNA with uridine-^{3}H, cultures were concentrated to 10^6 cells per milliliter using the same medium in which they had been growing; 5 μCi/ml of uridine-^{3}H (20 Ci/mmole) were then added. When indicated, 0.04 μg/ml of actinomycin was added to the concentrated culture 25 min before addition of the uridine-^{3}H.

RNA-DNA Hybridization and Hybridization-Inhibition

RNA extracted by the hot phenol–sodium dodecyl sulfate (SDS) method was hybridized with viral DNA according to Fujinaga and Green (1966). During alcohol precipitation of sucrose gradient regions, either 0.4 mg yeast RNA (Sigma Chemical Co., St. Louis, Mo.) or 0.2 mg phenol-purified KB cytoplasmic RNA was added as carrier.

Two sequential annealing reactions were performed to determine the relationship between Ad 2 RNA synthesized in the absence of actinomycin and Ad 2 RNA synthesized in the presence of actinomycin (0.04 μg/ml). A microtechnique was used in which viral DNA was immobilized on membranes 6.5 mm in diameter and hybridization was performed in 10 x 75 mm tubes. In step 1, 0.035 μg viral DNA was annealed with increasing amounts of unlabeled RNA in 0.15 ml of 2 $\times$ standard saline citrate (SSC) (SSC = 0.15 M NaCl–0.015 M Na_3 citrate) –0.1% SDS for 20 hr at 66°C. In step 2 of the hybridization-inhibition reaction, the RNA solution was removed and the DNA filter was rinsed twice with 1 ml of 2 $\times$ SSC; then the membrane was annealed for an additional 20 hr at 66°C with saturating amounts of labeled RNA in 0.15 ml of 2 $\times$ SSC –0.1% SDS.

Sucrose Gradient Analysis of Cytoplasmic Extracts

Crude cytoplasmic extracts were centrifuged through 7.5-45% gradients, 3 ¼ hr, 25,000 rpm, 5°C in the SW25.1 rotor, to obtain polysomes. When all ribosome species were to be obtained from an extract, the 7.5-45% gradients were formed on top of a 2 ml 65% sucrose cushion. After centrifugation for 16.5 hr at 20,000 rpm, 5°C, in the SW25.1 rotor, most of the polysomes were concentrated in the sucrose cushion and bottom of the gradient; 74S ribosomes and free subunits were resolved in the remainder of the gradient. Sucrose solutions were buffered with 0.01 M HEPES (*N*-2-hydroxyethylpiperazine-N′-2-ethane sulfonic acid, Calbiochem, Los Angeles, Calif.), pH 7.2, and contained 0.0015 M $MgCl_2$, 0.01 M NaCl. The ultraviolet absorbance pattern was determined by continuous recording at 260 mμ using a Gilford recording spectrophotometer. Trichloracetic acid–precipitable radioactivity of gradient fractions was determined by pipeting appropriate volumes onto Whatman 3MM filters and washing according to the method of Bollum (1959). All samples were counted in a liquid scintillation counter; the overlap of ^{14}C cpm into the ^{3}H channel was 20%. Less than 1% of the ^{3}H cpm was detected in the ^{14}C channel.

Cesium Chloride Density Gradient Analysis

Polysomes and 74S ribosomes were fixed with formaldehyde and banded in cesium chloride gradients essentially as described by Perry and Kelley (1966). Sucrose gradient fractions were treated for 18 hr with 5% formaldehyde. Formaldehyde solutions were adjusted to pH 7.2, 0.01 M HEPES, just before use. After formaldehyde treatment, the samples were dialyzed for 4–6 hr against 0.01 M HEPES, pH 7.2, 0.001 M $MgCl_2$ and mixed with a concentrated cesium chloride solution containing 0.01 M HEPES, pH 7.2, 0.001 M $MgCl_2$ to give a total volume of 4.5 ml. Centrifugation was carried out for 36 hr at 35,500 rpm, 5°C, in the SW39 rotor. Coating the cellulose nitrate tubes with bovine serum albumin (0.25% solution) yielded increased recovery from the gradients (70–90% of input cpm). Fractions of approximately 175 λ were collected and analyzed

for absorbancy at 260 mμ or for trichloroacetic acid–precipitable radioactivity (Bollum, 1959). Densities were determined from refractive index measurements.

Acrylamide Gel Electrophoresis

Polysomal RNA was analyzed on 2.8% acrylamide gels containing 10% glycerol (Loening, 1967; Bishop, Claybrook, and Spiegelman, 1967; Weinberg, Loening, Williams, and Penman, 1967). Polysome fractions were concentrated by treating with 0.5% SDS and coprecipitating with phenol-purified cytoplasmic KB RNA. After electrophoresis, gels were frozen, sliced, solubilized with alkali overnight, and counted in a scintillation counter as described previously (Okubo and Raskas, 1970). Of the input ^{14}C cpm, 10% were recorded in the ^{3}H channel; less than 1% of the ^{3}H cpm was recorded in the ^{14}C channel.

RESULTS

During productive infection of KB cells with Ad 2, viral DNA synthesis begins 6–7 hr after viral adsorption, and the accumulation of infectious virus in the nucleus occurs from 13 to 24 hr (Thomas and Green, 1969). 18 hr after infection viral RNA synthesis is maximal, and 80–100% of the 23 × 10^6 daltons of the Ad 2 genome are being transcribed (Fujinaga and Green, 1970).

Virus-Specific RNA Synthesized in Presence of Low Amounts of Actinomycin

The transport of viral RNA can be studied in the absence of ribosomal RNA synthesis. The viral infection reduces ribosome synthesis at least 80% by 18 hr after infection (Raskas, Thomas, and Green, 1970). Since low concentrations of actinomycin selectively inhibit ribosomal RNA synthesis (Perry, 1963; Roberts and Newman, 1966; Penman, Vesco, and Penman, 1968), we have added 0.04 μg/ml of actinomycin 25 min before addition of uridine-^{3}H to insure complete elimination of ribosome synthesis. The Ad 2 RNA's transcribed at 18 hr are high in guanine plus cytosine content (58–59%) (Thomas and Green, 1969), but viral RNA synthesis continues in the presence of the drug.

We have performed hybridization-inhibition experiments to determine whether adding actinomycin causes significant changes in the species of viral RNA transcribed. The saturation of 0.035 μg Ad 2 DNA with cytoplasmic RNA-^{3}H labeled 18–21 hr after infection in the presence or absence of actinomycin is shown in Fig. 1. The specific activity of RNA labeled in the presence of actinomycin is 30% less than that labeled in the absence of actinomycin (see legend to Fig. 1). Since the amount of viral RNA-^{3}H bound at saturation is reduced 50–60% when actinomycin is present (Fig. 1), there may be some selective reduction of Ad 2 RNA synthesis. For competition-inhibition experiments 2.8 × 10^5 cpm were used as saturation conditions for both the RNA labeled in the absence and the RNA labeled in the presence of actinomycin. Cold cytoplasmic RNA prepared from either infected cells treated for 3 ½ hr with actinomycin or from untreated cells harvested at the same time (21 hr) is equally capable of preventing the hybridization of either class of viral RNA-^{3}H (Fig. 2). The competitions were 90% effective in all four cases at the same concentration of unlabeled RNA, indicating that much the same viral RNA is made in the presence and absence of actinomycin.

We have also examined the size distribution of polysomal RNA-^{3}H synthesized in the presence of actinomycin (Fig. 3). The distribution of these RNA molecules closely resembles that obtained for viral RNA by fractionating polysomal RNA from untreated cells on acrylamide gels and then annealing with viral DNA (Parsons, Gardner, and Green, manuscript in preparation). Thus it is likely

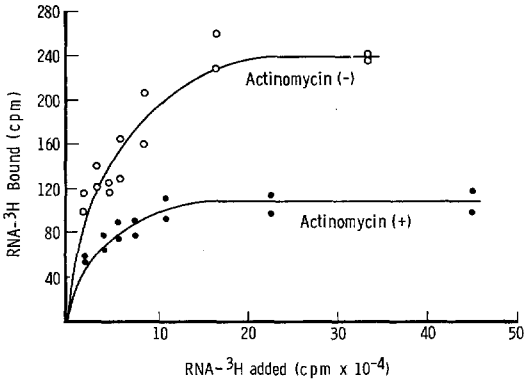

FIGURE 1 Saturation of Ad 2 DNA with cytoplasmic RNA-^{3}H labeled in absence or presence of 0.04 μg/ml actinomycin. Membrane filters containing 0.035 μg of DNA were incubated with increasing amounts of cytoplasmic RNA-^{3}H from Ad 2–infected KB cells labeled 18–21 hr after infection. Specific activity of the RNA labeled without actinomycin (open circles) was 2540 cpm/μg and that labeled in presence of actinomycin (closed circles) was 1780 cpm/μg.

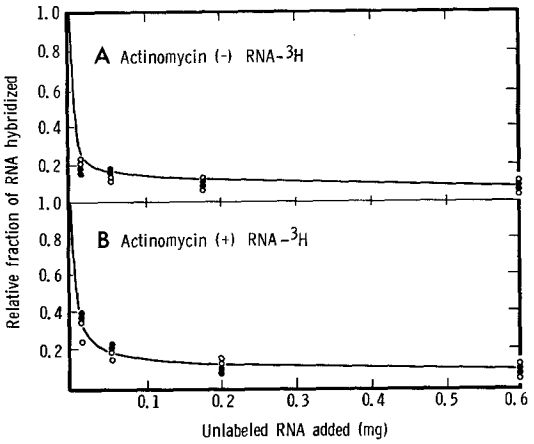

FIGURE 2 Hybridization-inhibition between Ad 2 RNA synthesized in absence or presence of 0.04 μg/ml actinomycin. Filters containing 0.035 μg of Ad 2 DNA were annealed for 24 hr with increasing amounts of cold cytoplasmic RNA from infected cells harvested at 21½ hr without actinomycin treatment (open circles) or after exposure to actinomycin for 3½ hr (closed circles). The filters were then incubated for an additional 20 hr with 2.8×10^5 cpm of cytoplasmic RNA-^{3}H from infected cells labeled at 18½ hr for 3 hr in the absence (A) or presence (B) of actinomycin.

that the addition of 0.04 μg/ml actinomycin leaves intact the pattern of viral RNA synthesis.

Polysome-Associated Viral RNA

Polysomal viral RNA synthesized 18 hr after infection is found in a wide range of polyribosome sizes. Cytoplasmic extracts prepared with nonidet P40 yield polysome distributions such as shown in Fig. 4. The polysomes from cells labeled 18–20 hr after infection were divided into three size groups, and the RNA extracted from each class was annealed to a large excess of viral DNA. From each size class of polyribosomes approximately 60% of the RNA-^{3}H annealed to viral DNA whether the RNA was labeled in the presence or absence of actinomycin (Table I). Since the cells used for this infection were uniformly labeled with uridine-^{14}C, we were able to calculate the amount of viral RNA per ribosome for each class of polysomes. As seen in Table I, viral mRNA's were equally loaded with ribosomes (^{3}H bound/^{14}C input) whether they were in large, medium, or small size polysomes.

Cytoplasmic extracts of mammalian cells often yield polysome preparations with cosedimenting ribonucleoprotein that contains rapidly labeled RNA. Such cosedimenting RNA is not a part of the polysome structure and is found when cells are broken mechanically (Penman, Vesco, and Penman, 1968) but not when they are disrupted by nonionic detergents such as Triton X-100 (Perry and Kelley, 1968). With nonidet P40, we obtain polysomes from Ad 2–infected cells that are free of significant amounts of cosedimenting ribonucleoprotein. Infected cells were labeled for 90 min at 18 hr after infection, and the isolated polyribosomes were sedimented to equilibrium in cesium chloride density gradients (Fig. 5 A). At most,

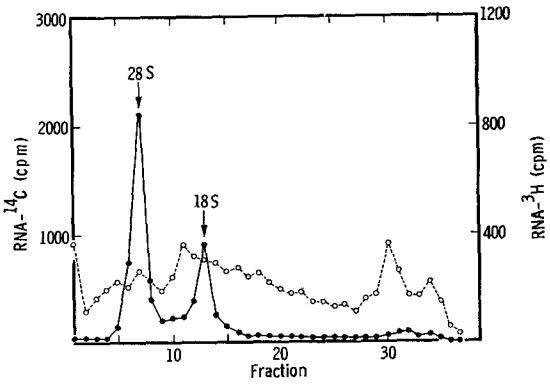

FIGURE 3 Acrylamide gel electrophoresis of polysomal RNA synthesized in the presence of actinomycin late after Ad 2 infection. Cells labeled before infection with uridine-^{14}C were exposed to uridine-^{3}H from 19 to 21 hr after infection in the presence of actinomycin (0.04 μg/ml); the polysomal RNA from extracts of these cells was analyzed on 2.8% acrylamide gels.

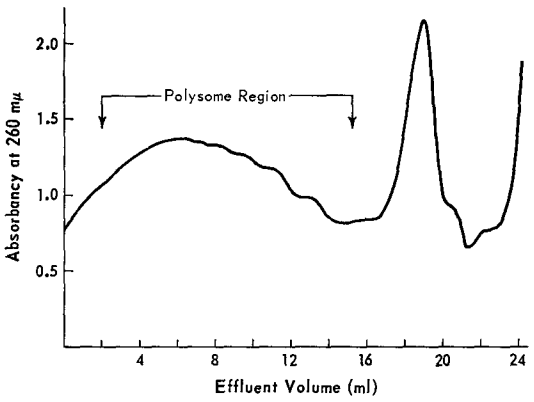

FIGURE 4 Polysome distribution obtained from KB cells 18 hr after infection with Ad 2. Cytoplasmic extracts from cells swollen in hypotonic medium were prepared with nonidet P40 and analyzed on 7.5–45% sucrose gradients as described in Methods.

TABLE I

Hybridization of RNA from Different Polysome Fractions

RNA sample		^{3}H cpm bound	Per cent of ^{3}H bound	^{3}H bound/^{14}C input
−Actinomycin	Large	514 530	63.5	0.65
	Medium	577 588	63.5	0.72
	Small	668 650	55.0	0.82
+Actinomycin	Large	325 371	58.5	0.44
	Medium	421 431	61.0	0.53
	Small	396 422	50.0	0.51

Infected cells that had been labeled with uridine-^{14}C for 24 hr before infection (as in Fig. 6) were exposed to uridine-^{3}H at 18 hr after infection for 2 hr in the absence or presence of 0.04 μg/ml actinomycin. Polysomes were obtained from sucrose gradients as in Fig. 4, and the polysomal RNA was annealed to 15 μg of viral DNA as described in Methods. The results from duplicate membranes are presented; less than 5 cpm were bound to membranes without DNA.

15% of the labeled RNA sedimenting with the ribosomes represents residual ribosome synthesis (Raskas, Thomas, and Green, 1970); the remainder is primarily viral mRNA (see Table I). Essentially all of the RNA-^{3}H had the same density as the polysomes, 1.53. In contrast, RNA-^{3}H cosedimenting with 74S ribosomes is composed of two distinct fractions (Fig. 5 B), one having the same density as the ribosomes and the other having the characteristic density of ribonucleoprotein, 1.43 (Perry and Kelley, 1968; Henshaw, 1968).

Kinetics of Viral RNA Transport

The kinetics of viral RNA transport from nucleus into total cytoplasm and polyribosomes were followed by the experimental procedure outlined in Fig. 6. Cells uniformly labeled with uridine-^{14}C were infected with Ad 2; with this procedure it is possible to quantitate amounts of uridine-^{3}H incorporated into viral RNA late in infection by comparison to the stable RNA-^{14}C. For all of these experiments 0.04 μg/ml of actinomycin was added 25 min before addition of uridine-^{3}H.

The relationship of cytoplasmic viral RNA to ribosomes was analyzed on sucrose gradients in which the polysomes were concentrated on a sucrose cushion and the 74S ribosomes and free subunits resolved in the remainder of the gradient (Fig. 7). The total cytoplasmic RNA-^{3}H is approximately 30% viral specific, and most of the nonviral RNA sediments at the top of the gradient (see below). Extracts from cells labeled for 65 min show a broad distribution of RNA-^{3}H beginning at about 10S and continuing throughout the gradient; there may be some accumulation of RNA-^{3}H in the polysomes. The increase in polysome-associated RNA is evident at 90 min and more so at 210 min. Compared to polyribosomes, the 74S ribosomes receive far less new RNA; this deficiency is twice as great as appears in the sucrose gradients because half of the RNA-^{3}H cosedimenting with the 74S ribosomes is cosedimenting ribonucleoprotein (Fig. 5).

The RNA from the polysome fractions and total cytoplasmic preparations was annealed to viral DNA to obtain the kinetics of viral RNA transport. Using a large excess of viral DNA, it is possible to deplete the solution of all viral RNA that can be

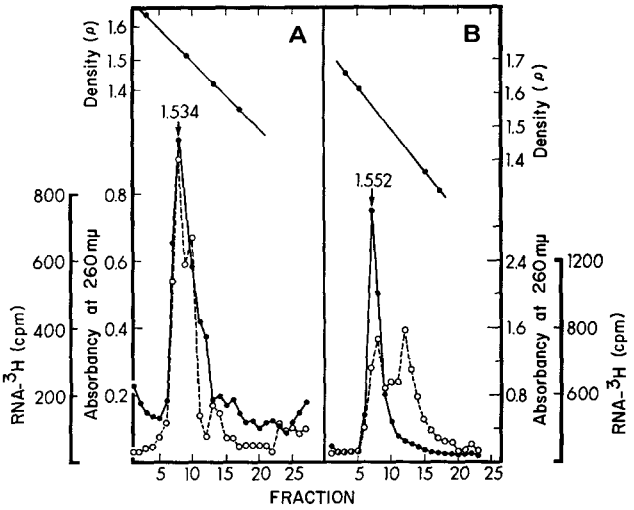

FIGURE 5 Density distribution of newly synthesized RNA cosedimenting with polysomes (A) and 74S ribosomes (B) 18 hr after Ad 2 infection. Infected cells were labeled with uridine-^{3}H for 90 min beginning 18 hr after infection; polysomes and 74S ribosomes were obtained from cytoplasmic extracts by sucrose gradient fractionation. The ribosome samples were treated with formaldehyde, were dialyzed, and were centrifuged to equilibrium in cesium chloride gradients as described in Methods. Optical density, closed circles; RNA-^{3}H cpm, open circles.

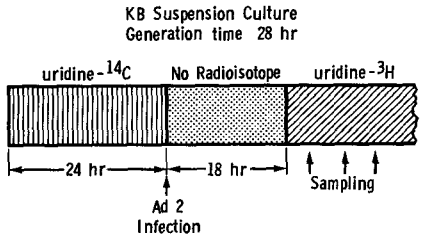

FIGURE 6 Procedure used in labeling cells for studies of Ad 2 RNA transport. In all instances 0.04 μg/ml actinomycin was added to the culture 25 min before uridine-^{3}H addition.

annealed in a one-step hybridization. The amount of newly synthesized viral RNA-^{3}H can then be normalized to the input RNA-^{14}C which remains constant during the labeling intervals late after infection. The annealing reactions used to obtain the kinetic data were performed under depletion conditions previously established; less than 1 μg of RNA was exposed to membranes having 10 or 15 μg of viral DNA. A sample of the results obtained (Table II) shows the increase of labeled viral RNA sequences in the total cytoplasm and in polysomes with longer labeling periods. From the annealing data we obtained the kinetics shown in Fig. 8. Labeled RNA-^{3}H is found in the cytoplasm 2 min after addition of the uridine-^{3}H, whereas viral RNA molecules can be detected in the cytoplasm at 10 min. The polysomal RNA includes new viral RNA-^{3}H molecules only after 30 min have elapsed.

Cytoplasmic Viral RNA that is not in Polysomes

The kinetic studies (Fig. 8) indicate that infected cells labeled for more than 10 min but less than 30 min have no polysomal viral RNA-^{3}H but do contain cytoplasmic viral RNA-^{3}H. That result was obtained when cells were labeled for 25 min (Fig. 9). The RNA-^{3}H sediments as three broad peaks, 60S, 20S, and 4S; essentially no newly synthesized RNA is in the polysomes. The 60S RNA-^{3}H and 20S RNA-^{3}H contain 37% and 18% viral RNA, whereas the low molecular weight RNA is only 3% viral specific. By 50 min viral RNA-^{3}H could be found in the polysomes as well as in the 60S and 20S fractions.

A significant fraction of the viral RNA in cytoplasmic extracts is not in polyribosomes even when cells are labeled for longer times. By comparing the amounts of polysomal and total cytoplasmic viral RNA, we calculate that 75% of the cytoplasmic viral RNA is not in polysomes even when cells are labeled for intervals ranging from 65 min to 150 min (Table III). A direct demonstration of

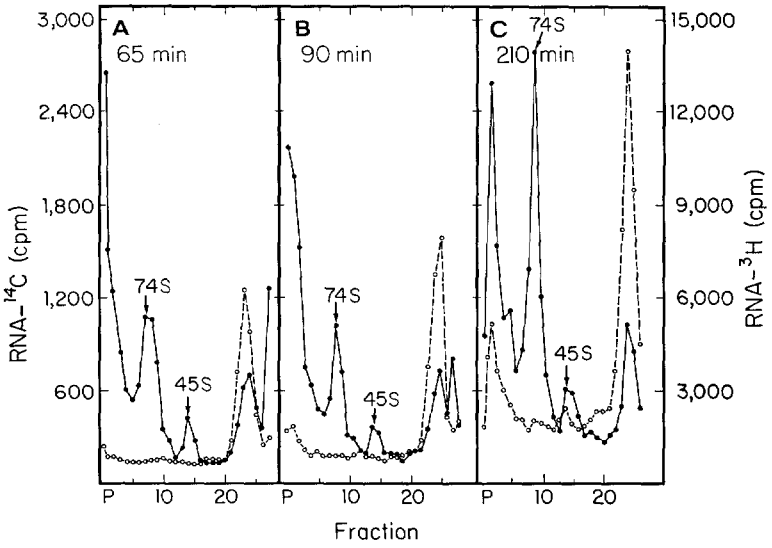

FIGURE 7 Sucrose gradient analysis of cytoplasmic extracts obtained from infected cells labeled beginning at 18 hr for 65 min (A), 90 min (B), and 210 min (C) in the presence of actinomycin (as shown in Fig. 6). Extracts prepared with nonidet P40 were centrifuged in the SW25.1 rotor through 7.5–45% sucrose gradients having a 2 ml 70% sucrose cushion; 100 lambda samples from the gradient fractions were sampled to determine the distribution of TCA-precipitable radioactivity. RNA-^{14}C, closed circles; RNA-^{3}H, open circles.

nonpolysomal cytoplasmic viral RNA was obtained by hybridizing RNA from cytoplasmic extracts of cells labeled for 210 min; the RNA sedimenting as 40S–55S was 37% viral specific (Table IV).

DISCUSSION

Synthesis of Viral RNA in Presence of Low Amounts of Actinomycin

For most experiments described here, we have used a low concentration of actinomycin (0.04 μg/ml) to complete the inhibition of ribosome synthesis (Perry, 1963; Roberts and Newman, 1966). The 25 min pretreatment with actinomycin before addition of uridine-^{3}H is sufficient to obtain full effect of the drug, for such treatment prevents any discernible synthesis of ribosomal RNA in HeLa cells (Penman, Vesco, and Penman, 1968). The addition of actinomycin apparently does not affect the pattern of viral RNA synthesis even though the late Ad 2 RNA's contain 58–59% guanine + cytosine. Under these conditions the polysome-associated RNA labeled for 2 hr (Table I) is at least 60% viral specific and has a sedimentation distribution (Fig. 3) that resembles the distribution of viral RNA molecules in polysomes (Parsons and Green, in preparation). This polysome-associated Ad 2 RNA is found in large, medium, and small size polysomes (Table I) and is not restricted to a single size 200S polysome as suggested for Ad 5–directed protein synthesis (Velicer and Ginsberg, 1968, 1970). Perhaps the cellular site of the RNA synthesis influences the effect of actinomycin treatment. Ribosomal RNA is synthesized in the nucleolus; presumably Ad 2 RNA is transcribed in the nucleoplasm.

Parameters of Viral RNA Transport and Association with Ribosomes

When uridine-^{3}H is added to a culture, labeled RNA is found in the cytoplasm within 2 min but viral sequences are not present until 10 min (Fig. 8). The nonviral cytoplasmic RNA-^{3}H present after very short labeling intervals is probably low molecular weight (Figs. 7 and 9) tRNA and pre-tRNA (Bernhardt and Darnell, 1969) and also 7S RNA, a species synthesized late after Ad 2 infection (Ohe, Weissman, and Cooke, 1969). Detectable amounts of viral RNA are found in polysomes at 30 min. To directly demonstrate that polysomes receive viral RNA some time after appearance of viral sequences in the cytoplasm, cells were labeled

TABLE II

Depletion Hybridization of Cytoplasmic and Polysomal Viral RNA

RNA sample	μg DNA	^{3}H cpm bound	^{3}H bound/^{14}C input	Per cent of input ^{3}H bound
20 min cytoplasm	10	165, 153	0.040	14
	15	196, 195		
	0	0, 2		
70 min cytoplasm	10	278, 252	0.202	31
	15	324, 303		
	0	3, 1		
120 min cytoplasm	10	389, 374	0.386	35
	15	446, 461		
	0	2, 2		
70 min polysomes	10	123, 125	0.109	65
	15	132, 132		
	0	0, 2		
150 min polysomes	10	377, 408	0.271	61
	15	371, 392		
	0	0, 0		

Infected cells prelabeled with uridine-^{14}C were labeled with uridine-^{3}H as outlined in Fig. 6; RNA was obtained from the total cytoplasm or from the polysomal fractions of gradients such as shown in Fig. 7. Annealing reactions of purified RNA were carried out for 20 hr at 66°C. The results of duplicate membranes are presented. In calculating the ratio ^{3}H bound/^{14}C input, the ^{3}H cpm were the average of the cpm bound to the 15 μg membranes; the ^{14}C input was the ^{14}C-cpm added to the reaction vial.

for 25 min (Fig. 9); in such extracts, viral RNA was found in the cytoplasm but not in polysomes. The 30 min time of entry of viral RNA into polysomes agrees with a calculated entry time for HeLa cell messenger into polysomes (Penman, Vesco, and Penman, 1968); the latter result was derived from sedimentation analysis of pulse-labeled polysomal RNA synthesized in the presence of actinomycin.

The Role of 45S Ribosome Subunits in Messenger RNA Transport and Polyribosome Formation

The results described above demonstrate that mRNA transport can occur in the absence of ribosome synthesis. One possible first step in transport is the formation of a complex between free 45S ribosome subunits and mRNA. If the 45S subunit is required for transport, it must function as a cytoplasmic cofactor, for newly synthesized nuclear precursors to 45S ribosomes must be absent in Ad 2–infected cells treated with actinomycin. In our analysis of cytoplasmic extracts we fail to find a significant peak of viral RNA sedimenting at 45S as had been previously reported for messenger-like RNA in other systems (Joklik and Becker, 1965 *a*, *b*; Henshaw, Revel, and Hiatt, 1965; McConkey and Hopkins, 1965). Rather, we find viral RNA distributed throughout the sucrose gradient; 45S-mRNA complexes may be absent or in very low concentrations in our extracts, or the 45S-mRNA complexes may have a wide diversity of sedimentation values and not be detected as 45S particles.

Mammalian cells may contain 45S subunit–mRNA complexes that are not directly related to mRNA transport. If ribosome metabolism in mammalian cells is similar to that in bacteria (Mangiarotti and Schlessinger, 1967; Kaempfer, Meselson, and Raskas, 1968; Okubo and Raskas, 1970), new polysomes would be formed by the initial association of mRNA with the small ribosome subunit (Nomura and Lowry, 1967; Nomura, Lowry, and Guthrie, 1967; Hoerz and McCarty, 1969; Holder and Lingrel, 1970). Such 45S-mRNA

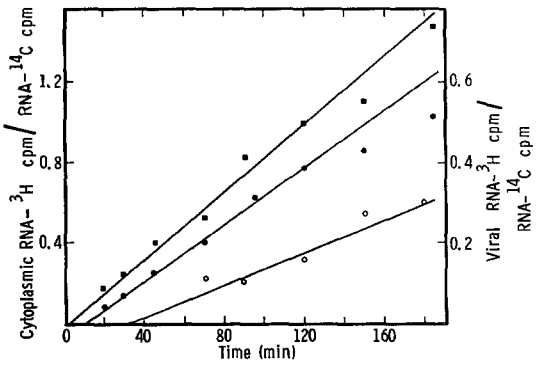

FIGURE 8 Kinetics of RNA transport. Infected cells labeled for different intervals as shown in Fig. 6 were treated with nonidet P40 to obtain the total cytoplasmic extract and fractionated on sucrose gradients to obtain polysomes (Fig. 7). The kinetics of appearance of RNA-^{3}H in the cytoplasm were obtained from the values of (^{3}H/^{14}C) for total cytoplasmic RNA at different times (closed squares). The accumulation of viral RNA sequences in the total cytoplasm (closed circles) and the polysomes (open circles) was determined by annealing RNA samples to a large excess of viral DNA and calculating the increasing amounts of viral RNA-^{3}H as compared to the stable RNA-^{14}C in the preparation. Samples of these data are given in Table II. The slope of the line drawn for polysomal viral RNA is based on a least-squares calculation.

complexes could be formed subsequent to transport and be unrelated to the mechanism of transport.

Nonpolysomal Cytoplasmic RNA

Whether cells are labeled for very short times (25 min) (Fig. 9) or for periods as long as 150 min (Tables III and IV), more than 70% of the cytoplasmic viral RNA is not in polyribosomes. We can suggest a number of possibilities for the origin or role of the nonpolysomal viral RNA:

(*a*) The nonpolysomal cytoplasmic viral RNA may be a pool of mRNA which serves as precursor to polysomal mRNA.

(*b*) The nonpolysomal cytoplasmic RNA may be an artifact of late-infected cells; there may be excessive unregulated transcription of viral RNA which reaches the cytoplasm but never enters polyribosomes. Experiments are in progress to determine if nonpolysomal cytoplasmic viral RNA is present early after infection with Ad 2 and in cells transformed with Ad 2.

(*c*) Late Ad 2–RNA metabolism (Parsons, Gardner, and Green, in preparation) includes a processing step in which large molecules are cleaved (Penman, Scherrer, Becker, and Darnell, 1963; Scherrer, Latham, and Darnell, 1963). The nonpolysomal cytoplasmic RNA may represent the portion of processed RNA which will not be utilized for translation. If such is the case, the nonpolysomal and polysomal RNA's should contain different nucleotide sequences. This possibility is being analyzed by experiments on competition-inhibition between cytoplasmic nonpolysomal and polysomal RNA.

(*d*) We think it unlikely that most of these nonpolysomal RNA molecules have leaked from late-infected nuclei, for the per cent of the cytoplasmic RNA in polysomes remains constant (Table III) during a period in which the distribution of RNA-^{3}H between nucleus and cytoplasm is rapidly changing.

The in vivo form of the nonpolysomal viral RNA is unknown. Whether the RNA is free of protein, is associated with special proteins related to transport (Spirin, Belitsina, and Ajtkhozhin, 1964; Spirin

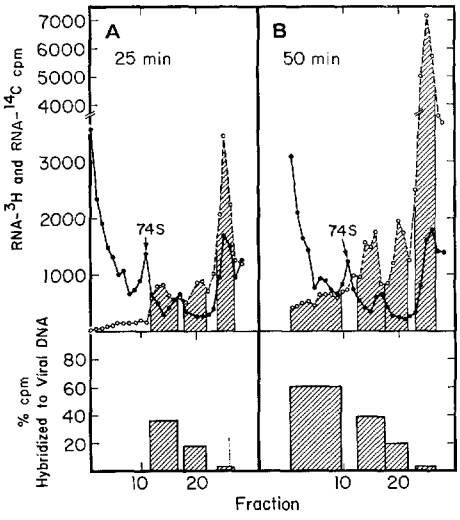

FIGURE 9 Sedimentation distribution of RNA-^{3}H in cytoplasmic extracts labeled for 25 min or 50 min. Cytoplasmic extracts from infected cells labeled with uridine-^{3}H for 25 min (A) or 50 min (B) were analyzed by sucrose gradient centrifugation; the fractions indicated were pooled, extracted, and annealed to excess viral DNA. The per cent of the RNA-^{3}H in each grouping (polysomal, 60S, 20S, or 4S) that hybridized to viral DNA is shown in the lower portion of each panel. 100 lambda samples from the gradient fractions were sampled to obtain the TCA-precipitable distribution of RNA-^{14}C (closed circles) and RNA-^{3}H (open circles).

TABLE III

Per Cent of Cytoplasmic Viral RNA Sequences in Polysomes 18 Hr after Infection

Experiment	Time labeled with uridine-^{3}H	(A) Polysomal ^{3}H viral RNA/^{14}C RNA	(B) Cytoplasmic ^{3}H viral RNA/^{14}C RNA	(C) Per cent of ^{14}C RNA in polysomes	(D) Per cent of cytoplasmic viral RNA in polysomes
	min				
I	70	0.106	0.202	45	23.6
I	150	0.229	0.427	39	21.1
II	65	0.123	0.261	47	22.1
II	90	0.192	0.348	51	28.1
II	150	0.288	0.694	43	17.8

The values of ^{3}H viral RNA/^{14}C RNA (A and B) were from experiments such as those presented in Table II. The per cent of the RNA-^{14}C in polysomes (C) was calculated from sucrose gradient analyses of ribosomes as in Fig. 7. The per cent of cytoplasmic viral RNA in polysomes (D) is the ratio (A)/(B) multiplied by (C).

TABLE IV

Viral RNA Content of Nonpolysomal Cytoplasmic RNA Labeled for 210 Min

RNA sample	μg DNA	^{3}H cpm bound	Per cent of ^{3}H bound
40-55S	10	145, 121	37.2
	15	105, 153	
	—	0, 1	
4-6S	10	154, 153	4.0
	15	184, 256	
	—	0, 3	

Cytoplasmic extracts of late-infected cells labeled for 210 min in the presence of actinomycin were fractionated on sucrose gradients as in Fig. 7. The RNA from the 40-55S and 4-6S regions of the gradient shown in Fig. 7 C was extracted and hybridized to Ad 2 DNA as described in Methods. The results from duplicate membranes are presented.

and Nemer, 1965; Infante and Nemer, 1968; Henshaw and Loebenstein, 1970), or is nonspecifically aggregated with cytoplasmic proteins (Girard and Baltimore, 1966) remains to be determined.

Messenger RNA and 74S Ribosomes

Single ribosomes are approximately fivefold deficient in receiving newly synthesized messenger-like RNA as compared to polysomes (Fig. 7). This observation agrees with the early report that 80S ribosomes isolated from rat liver contain less nascent protein than polyribosomes (Noll, Staehelin, and Wetstein, 1963). Other observations indicate that a fraction of the 74S ribosomes present in extracts of mammalian cells is a unique ribosome species: In exponentially growing KB cells new subunits preferentially enter polysomes rather than 74S ribosomes (Okubo and Raskas, 1970; see also Girard, Latham, Penman, and Darnell, 1965; Joklik and Becker, 1965 *a*; Kabat and Rich, 1969). Conditions that decrease the rate of protein synthesis in ascites tumor cells do not affect the size of the free subunit fraction, but cause an increase in the per cent of ribosomes found as single ribosomes (Hogan and Korner, 1968); and single ribosomes, in contrast to polyribosomes, are susceptible to dissociation into subunits by high salt treatment (Vaughan, Warner, and Darnell, 1967; Zylber and Penman, 1970). Presumably there are two kinds of monomers in mammalian cells. A fraction of the 74S ribosomes would contain the ribosome-bound messenger RNA and nascent proteins detected in the 74S region of sucrose gradients. The remainder of the 74S particles are inactive in protein synthesis and may represent a storage form of ribosomes.

It is interesting to speculate that messenger-deficient 74S ribosomes and a pool of cytoplasmic nonpolysomal RNA interact to give translational regulation in mammalian cells. Thus the nonpolysomal viral RNA in the cytoplasm of adenovirus-infected KB cells and the nonpolysomal heterogeneous cytoplasmic RNA of HeLa cells (Attardi, and Attardi, 1967; Penman, Vesco, and Penman, 1968) may have similar functions: Some mRNA species may be stored in the cytoplasm; when needed, these mRNA's could associate with ribosome subunits derived from 74S particles.

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