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Procedure for the Preparation of Milligram Quantities of Adenovirus Messenger Ribonucleic Acid

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Late after adenovirus 2 infection (18 hr), nearly all newly synthesized polysomal messenger ribonucleic acid (mRNA) is viral specified. Large amounts of adenovirus mRNA have been purified by utilizing membrane filtration at high ionic strength. With this procedure, molecules that contain polyadenylic acid [poly (A)] tracts are bound selectively, and ribosomal RNA can be separated from the viral mRNA which contains poly(A). Polysomal RNA synthesized 18 hr after infection was labeled in the presence of 0.02 μg of actinomycin D per ml and extracted at *p*H 9.0. This RNA annealed 40% to 3 μg of adenovirus 2 deoxyribonucleic acid; the RNA selected by membrane filtration bound 80% under the same conditions. The RNA eluted from membrane filters was 80 to 90% greater than 18*S* and contained species migrating as 31, 27, and 24*S*. Binding of polysomal RNA to individual membrane filters was linear, using as much as 300 μg of RNA per membrane. A 1.1-mg amount of viral RNA was prepared from 17.7 mg of polysomal RNA that had been purified by extraction at *p*H 9.0.

Most polysomal messenger ribonucleic acid (mRNA) in eukaryotic cells contains covalently linked adenylic acid [poly(A)] tracts of 150 to 200 nucleotides (4, 5, 11). Similar poly(A) tracts are found in RNAs specified by three deoxyribonucleic acid (DNA) viruses, vaccinia virus (9, 22), adenovirus 2(15), and simian virus 40 (24), as well as in the RNA genome of RNA tumor viruses (6, 7, 10), poliovirus (1, 26), and Sindbis virus (8).

Although the function of poly(A) sequences in vivo is not known, this property can be used to purify milligram quantities of mRNA. At high ionic strength, molecules that contain poly(A) sequences bind to cellulose nitrate filters (Millipore Corp.) (11). Thus polysomal mRNA can be separated from structural ribosomal RNA. We have utilized this method to purify viral RNA present in polyribosomes late after infection with adenovirus 2. Such preparations should be useful in the structural and functional analysis of viral mRNA.

MATERIALS AND METHODS

Cell culture, virus infection, and labeling. KB cells were grown in suspension cultures and infected with adenovirus 2 as previously described (17, 18). At 16 hr after infection, cultures were concentrated three times in fresh medium containing 0.02 μg of actinomycin D per ml. After 30 min, ^{3}H-uridine or ^{3}H-adenosine (0.5 to 2 μCi/ml; 20 Ci/mmole) was added, and cultures were harvested 3.5 hr later. To obtain radioactive ribosomal RNA, uninfected cultures were labeled for 18 hr with ^{14}C-uridine (0.5 μCi/ml).

Preparation of polyribosomes and RNA. Cultures were harvested by pouring over crushed frozen phosphate-buffered saline (PBS) lacking Mg^{2+} and Ca^{2+} (18). Cell pellets were washed twice with PBS, swollen in five volumes of RSB [0.01 M tris(hydroxymethyl)aminomethane (Tris) (*p*H 7.4), 0.01 M NaCl, 0.0015 M $MgCl_2$], and disrupted by 10 to 15 strokes with a Dounce homogenizer (14). The homogenate was then centrifuged at 30,000 × *g* for 30 min in the Sorvall RC2-B centrifuge, and the supernatant fraction (S-30 fraction) was decanted carefully. Polyribosomes were isolated from the S-30 fraction by centrifugation (2.5 hr in the Ti50 rotor at 45,000 rev/min) through a 1 M sucrose solution containing 0.05 M Tris(*p*H 7.6), 0.025 M KCl, 0.005 M $MgCl_2$ (TKM). The polysome pellet was resuspended in TKM buffer, and RNA was extracted at *p*H 9.0 as described by Lee et al. (11).

Selection and characterization of polysomal RNA components containing poly(A). RNA preparations were resuspended in 0.5 M KCl, 0.001 M $MgCl_2$, 0.01 M Tris (*p*H 7.6), and molecules containing poly(A) tracts were obtained by adsorption to membrane filters (11). Bound RNA was eluted from membrane filters by incubation at 0 C in 10^{-3} M Tris (*p*H 7.6) for two sequential 1-hr intervals. The elution buffer contained 0.25% sodium dodecyl sulfate (SDS) ex-

cept when the preparation was used for RNA quantitation by the orcinol method (21).

RNA was annealed to viral DNA as previously described (16). Electrophoresis of RNA on 2.8% polyacrylamide gels was carried out for 3.5 hr (17). The ribonuclease-resistant fraction of various RNA preparations was determined by using both T_1 and pancreatic ribonuclease as described by Kates (9).

RESULTS

Late after adenovirus 2 infection (18 hr), viral genes dominate cellular synthetic activity. At least 80% of the proteins synthesized are viral structural polypeptides (20, 25), and a minimum of 60% of the newly synthesized polysomal RNA is viral specific (17). Since nearly all the polysomal mRNA is viral coded, isolation of functional viral mRNA requires the separation of ribosomal RNA from viral polysomal RNA.

Binding to membrane filters of polysomal mRNA synthesized late after infection. By 18 hr after adenovirus 2 infection, ribosome synthesis is inhibited at least 80% (18). If low amounts of actinomycin D are added, the suppression of ribosomal RNA synthesis is further enhanced, but viral RNA synthesis continues (17). Therefore, to label polysomal mRNA, ^{3}H-uridine was added to cultures 17 to 20 hr after infection in the presence of 0.02 μg of actinomycin per ml. Polyribosomes were prepared as described in Materials and Methods, and polysomal RNA was extracted with phenol either at *p*H 9.0 or *p*H 7.6. As reported by Lee et al. (11), extraction of polyribosomes at neutral *p*H results in the preferential loss of poly(A) tracts and molecules containing poly(A) sequences. The molecules obtained from the *p*H 9.0 extraction bound to membrane filters 40%, whereas those prepared by *p*H 7.6 extraction were bound only 7% (Table 1). Other preparations of RNA bound as much as 60% after extraction at *p*H 9.0. As a control, ribosomal RNA from uninfected cells was extracted at both *p*H 9.0 and *p*H 7.6. In agreement with earlier results (11), the yield of ribosomal RNA was decreased by extracting at *p*H 9.0, and the ribosomal RNA preparations bound less than 1% to membrane filters. Thus the adenovirus mRNA synthesized late after infection can be separated from the bulk of ribosomal RNA by extraction at *p*H 9.0 followed by preferential adsorption to membrane filters at high salt concentrations.

Characterization of RNA bound to membrane filters. The polysomal RNA labeled late after infection was extracted at *p*H 9.0 and analyzed on polyacrylamide gels (Fig. 1A). The same sample was passed through a membrane filter, and the RNA bound was eluted, concentrated by EtOH precipitation, and reanalyzed by elec-

TABLE 1. *Binding of polysomal RNA to membrane filters*[a]

RNA source	Counts/ min input	Counts/ min bound	Percent bound
*p*H 7.6 extraction	7,672	524	7
*p*H 9.0 extraction	11,968	4,755	40

[a] At 16 hr after infection with adenovirus 2, 0.02 μg of actinomycin D per ml was added, and 30 min later the cultures were labeled with ^{3}H-uridine for 3.5 hr as described in Materials and Methods. Polyribosomes were prepared and polysomal RNA was extracted at *p*H 7.6 or *p*H 9.0 and then tested for ability to bind to membrane filters as described by Lee et al. (1).

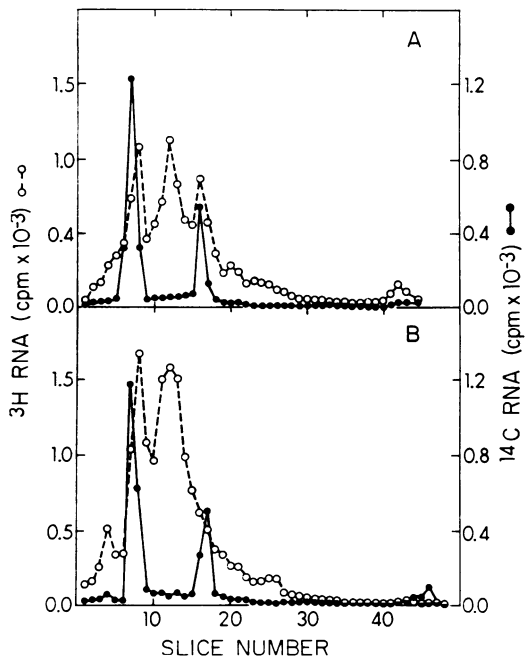

FIG. 1. *Polyacrylamide gel electrophoresis of polysomal RNA. Cultures infected with adenovirus 2 for 16 hr were treated with 0.02 μg of actinomycin D per ml for 30 min and then labeled with ^{3}H-adenosine (2 μCi/ml) for 3.5 hr as described in Materials and Methods. Polyribosomes were prepared by centrifugation through 1 M sucrose in TKM buffer, and polysomal RNA was extracted at pH 9.0. A portion of the polysomal RNA was bound to membrane filters, eluted, and concentrated by alcohol precipitation. A, size distribution of RNA extracted at pH 9.0; B, size distribution of RNA eluted from membrane filters. In both instances, ^{14}C-ribosomal RNA was added to provide reference markers. Symbols: ——●——●——, ^{14}C-RNA; - - -○- - -○- - -, ^{3}H-RNA.*

trophoresis. The RNA eluted from the membranes was not degraded as compared to the initial preparation (Fig. 1B); in fact, the preparation was enriched considerably with the higher-molecular-weight species (20-30*S*). Of the RNA extracted at *p*H 9.0, 40% hybridized with viral DNA (Table 2). The RNA eluted from the membrane filter annealed 80%.

Large-scale preparation of viral mRNA. A single membrane filter can bind substantial amounts of viral RNA perpared by *p*H 9.0 extraction of polyribosomes. By using a preparation labeled late after infection, the amount of RNA bound was proportional to the input polysomal RNA over a wide range of concentrations (Fig. 2). The membrane was not saturated when using as much as 300 μg per filter. When large amounts of RNA were bound to membranes, eluted, and collected by alcohol precipitation, the final yield was approximately 6.2% of the RNA obtained by extraction; for example, using nine membrane filters 1.1 mg of RNA was obtained from 17.7 mg. (RNA was prepared by *p*H 9.0 extraction of polyribosomes purified from KB cultures 18 hr after adenovirus 2 infection. The RNA was adsorbed on nine membrane filters and eluted in the absence of SDS).

DISCUSSION

As shown by the experiments described here, the poly(A) tracts of adenovirus RNA enable the large-scale purification of the viral mRNA in polyribosomes. The procedure we have described here does not require a hybridization step prior to the membrane binding. Since structural viral proteins account for nearly all polypeptide synthesis late after infection, most polysomal mRNA must be viral in origin. Polysomal mRNA that was synthesized late after infection annealed to adenovirus DNA 40%

TABLE 2. *Hybridization of late polysomal RNA to adenovirus DNA before and after binding to membrane filters*[a]

RNA source	Counts/min input	Counts/min hybridized	Percent hybridization
Polysomal	1,908	828	40
Eluted	1,412	1,192	80

[a] Polysomal RNA from infected cultures was extracted at *p*H 9.0 as described in Materials and Methods and in Table 1. The RNA obtained by extraction at *p*H 9.0 was passed through a membrane filter and then eluted in 0.001 M Tris (*p*H 7.6) containing 0.25% SDS. The total preparation and the eluted RNA were each annealed to membranes containing 3 μg of adenovirus 2 DNA.

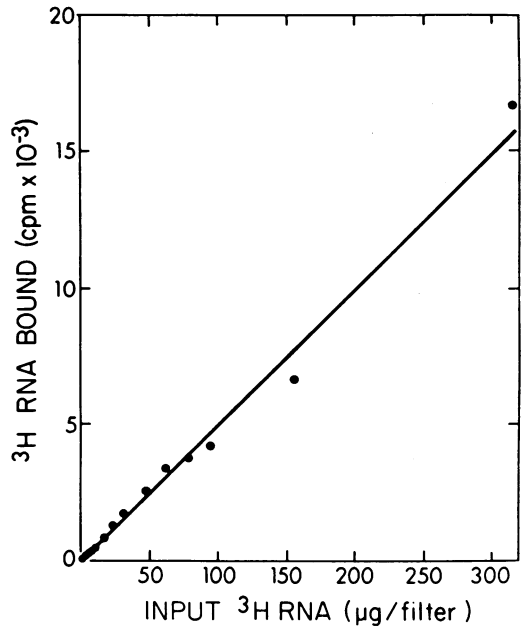

FIG. 2. *Binding capacity of membrane filters for polysomal adenovirus RNA. Polysomal mRNA synthesized late after infection was labeled with ^{3}H-uridine and purified as described in the legend to Fig. 1 and in Materials and Methods. Increasing amounts of ^{3}H-RNA were absorbed to individual membrane filters, and the counts per minute bound was determined.*

prior to binding, and 80% after elution from membrane filters (Table 2). When labeled with ^{3}H-adenosine, the total polysomal RNA was 10% resistant to pancreatic and T_1 ribonuclease. After purification by membrane filtration, the RNA was 17% resistant (*unpublished data*). This observation and the loss of the 18*S* peak (Fig. 1) during binding are consistent with the possibility that there may be some residual ribosomal RNA synthesis in the labeling conditions used here. Alternatively, the purified RNA eluted from the membrane may hybridize more efficiently.

The eluted RNA included species migrating as 31, 27, and 23*S* and lacked some of the 18*S* RNA and other smaller molecules present in the total polysomal RNA (Fig. 1b). A comparison of the individual viral RNA species synthesized in vivo (13) and those eluted from membrane filters is necessary before we can conclude with certainty that intact mRNA is obtained by membrane filtration. In addition, it should be noted that the stability of viral RNA in vivo is not known, and polyribosomes isolated 18 hr after infection may still contain viral RNA synthesized earlier in the infection. Therefore the

size distribution of the polysomal mRNA transcribed late after infection may not be representative of the total polysomal mRNA present late in the infection.

Other laboratories have reported the preparation of mRNA from mouse sarcoma 180 ascites cells (3), and the mRNAs for hemoglobin (2), myeloma (23), and ovalbumin and avidin (12, 19) proteins by using the poly(A) tracts as a basis of purification. The latter preparations were shown to be functionally active in in vitro protein-synthesizing systems. Thus it is likely that binding to a matrix that adsorbs poly(A) sequences will prove to be useful as a general method of preparing mRNA for functional and biochemical analysis. Most, if not all, the adenovirus mRNA species must be present in the RNA eluted from membrane filters, for 80% or more of the polysomal adenovirus RNA synthesized late after infection contains poly(A) tracts (15). Since the mRNA purified as described here is essentially all virus specified (Table 2), it can be tested for the ability to stimulate the in vitro synthesis of viral polypeptides.

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