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Cell-Free Translation of Highly Purified Adenovirus Messenger RNA

(polysomal RNA/oligo(dT)–cellulose/RNA·DNA hybridization/Krebs II ascites system)

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ABSTRACT **Several polypeptides contained in the coat and the core of adenovirus type 2 particles have been identified as virus gene products. They are synthesized *in vitro* by a cell-free protein-making system programmed by adenovirus type 2 messenger RNA that has been hybridized with and eluted from virus DNA. Fractionation by size yields subpopulations of viral messenger RNA that differ in their coding specificities. The procedures outlined in this study may be used to establish a genetic map of adenovirus type 2.**

Amber and deletion mutants provide useful tools for the identification of viral proteins because alterations in the affected gene products can readily be recognized in sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel electropherograms. Unfortunately, selection procedures for either type of mutant in adenoviruses are presently not available. This has prompted us to use an alternate method for the analysis of adenovirus gene functions.

We examined the coding capacity of mRNA extracted from polysomes of adenovirus type 2 (ad_2)-infected cells in a cell-free translation system derived from Krebs II ascites cells. In earlier reports (1, 2), we showed that this RNA directs cell-free synthesis of ad_2 coat and core polypeptides. In order to prove that these are indeed virus gene products, we found it necessary to thoroughly purify viral messenger RNA. This study will demonstrate that highly purified viral mRNA can be obtained from the infected cells, and that the RNA (like the less pure preparations used in our earlier experiments) can direct cell-free synthesis of major virion polypeptides.

METHODS

Propagation of KB Cells in spinner culture, virus strain, mode of infection, pulse-labeling of RNA with [^{3}H]uridine 16–18 hr after infection, phenol-extraction of RNA, as well as the composition of a cell-free translation system from Krebs II ascites cells, have been described elsewhere (1, 2).

Purification of mRNA. A total of 100 mg of polysomal RNA at a concentration of 1 mg/ml in adsorption buffer (10 mM Tris·HCl, pH 7.5–0.5 M KCl) was applied at room temperature at a flow rate of about 60 ml/hr to a 5 × 2.5-cm oligo(dT)–cellulose column (3). The column was washed with adsorption buffer until all nonadsorbed material was removed. Adsorbed RNA was eluted with 10 mM Tris·HCl, pH 7.5, adjusted to 0.1 M KCl, and precipitated overnight with 2.5 volumes of ethanol at −20°. Ad_2 DNA, either ^{14}C-labeled or unlabeled, was prepared from purified virions (4), sheared by sonication (1) to an average fragment size of 10^5 daltons, denatured by 10 min of boiling (followed by rapid cooling), and annealed to RNA that had been eluted from oligo(dT)–cellulose. The reaction mixture (10 ml) contained 200 μg/ml of DNA, 50 μg/ml of RNA, 2 M urea (Schwarz/Mann, ultrapure) (5), 0.2 M LiCl, 0.24 M sodium phosphate (pH 6.0), and 0.1% (w/v) $NaDodSO_4$. After 20 hr of incubation at 37°, the assay was adjusted to 8 M urea. Twelve milliliters of reaction mixture were applied to a 5 × 2.5-cm column of hydroxyapatite (Biogel HTP, DNA-grade, BioRad), equilibrated with 0.24 M sodium phosphate (pH 6.8)–8 M urea–0.1% (w/v) $NaDodSO_4$ (6), at room temperature at a constant flow rate of 24 ml/hr. Nonhybridized RNA was eluted in two bed volumes of equilibration buffer minus $NaDodSO_4$. The column was washed with one bed volume of 0.14 M sodium phosphate, pH 6.8, to remove urea. DNA·DNA and RNA·DNA hybrids were eluted with three bed volumes of 0.4 sodium phosphate, pH 6.8, and dialyzed against 50 volumes of 0.15 M NaCl–10 mM Tris·HCl, pH 7.2–0.1 mM EDTA, first at room temperature for 1 hr to prevent precipitation of phosphate salt in the dialysis tubing, then at 4° for another 12 hr. The material was precipitated with ethanol, resuspended in 10 mM Tris·HCl, pH 7.2, at a concentration of 10 mg of nucleic acid per ml, and boiled 1 min to denature hybrid structures. Ribosomal RNA was not degraded under similar conditions. After it was rapidly cooled, the material was sedimented through a linear gradient of 15–30% (w/v) sucrose (Schwarz/Mann, ultrapure) in 10 mM Tris·HCl, pH 7.5–0.1 M NaCl–0.1 mM EDTA, at 55,000 rpm in cellulose nitrate tubes in the Spinco SW56 rotor for 3 hr at 4°. Up to 500 μg of nucleic acid were applied to one centrifuge tube. Fractions containing DNA-free RNA were pooled, and concentrated by ethanol precipitation.

RESULTS

Purification of ad_2 mRNA. Pulse-labeled RNA was extracted from polysomes of KB cells that were actively engaged in virion protein synthesis (1). Poly(A)-containing mRNA was selected by adsorption of crude extracts to oligo(dT)–cellulose (3). Nearly all of the radioactivity of the preparation annealed with ad_2 DNA under conditions of exhaustive hybridization (Table 1). The experiment indicated that most of the newly synthesized polysomal mRNA was of viral origin, but did not, examine the nature of unlabeled RNA. Since biologically active host cell mRNA was likely to be contained in the preparation as well (1, 7), viral mRNA was

Abbreviations: ad_2, adenovirus type 2; $NaDodSO_4$, sodium dodecyl sulfate; cDNA, DNA synthesized *in vitro* on mRNA templates.

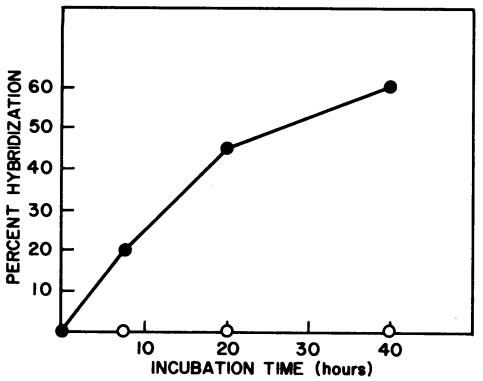

FIG. 1. Kinetics of hybrid formation between ad_2 DNA and mRNA. Pulse-labeled polysomal RNA from infected (●) or mock-infected (○) cells was purified by oligo(dT)–cellulose chromatography, and hybridized with unlabeled, single-stranded DNA fragments in a 4-ml reaction mixture. At the indicated times, 1-ml aliquots were withdrawn from the hybridization assay, and hybrid formation was determined by hydroxyapatite chromatography in the presence of urea (see *Materials and Methods* for details). RNA·DNA hybrids were precipitated with trichloroacetic acid on Millipore filters. Radioactivity was determined in a toluene-based scintillation fluid.

further purified by preparative hybridization with sheared, denatured ad_2 DNA. Annealing occurred at reduced temperature, and the time of incubation was limited to protect the integrity of viral mRNA. Up to 60% of radioactive RNA participated in hybrid formation under these conditions (Fig. 1). Nonhybridized RNA was removed by hydroxyapatite chromatography in the presence of urea (see *Materials and*

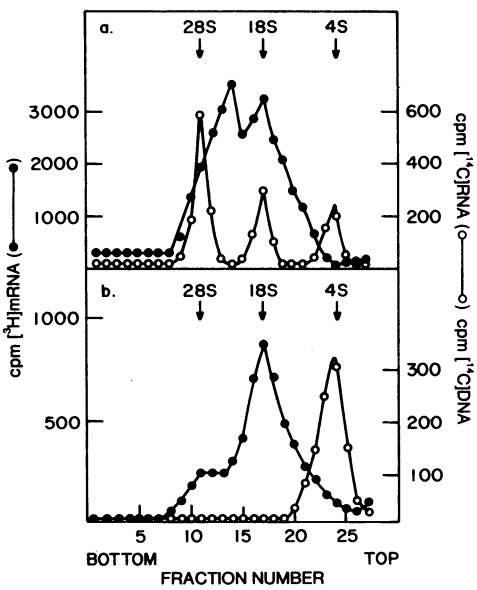

FIG. 2. Sedimentation of ad_2 mRNA through gradients of sucrose in neutral aqueous solution. Two gradients were prepared as indicated in *Materials and Methods*. [³H]RNA, purified on oligo(dT)–cellulose, and [¹⁴C]rRNA (added as a size reference) were layered onto one gradient (*a*). Hybrids of [³H]RNA and [¹⁴C]DNA were denatured and applied to the other gradient (*b*). The gradients were centrifuged in parallel, and fractions were collected from the bottom of the centrifuge tubes. Aliquots of each fraction were diluted 10-fold with water and counted in Aquasol (New England Nuclear Corp.).

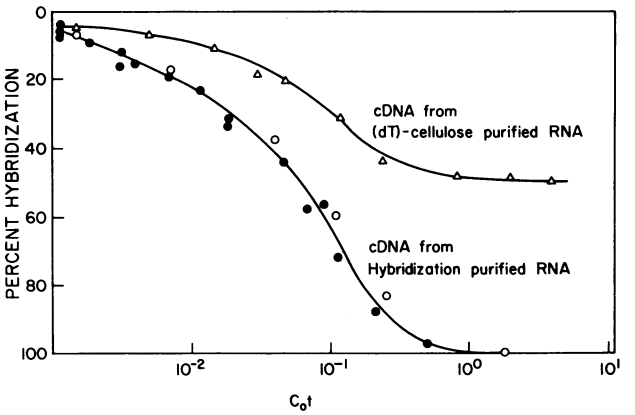

FIG. 3. Reassociation kinetics of cDNA and virion DNA. DNA complementary either to RNA purified on oligo(dT)–cellulose, derived from ad_2-infected cells (△), or to ad_2 RNA that had been further purified by preparative hybridization (○), was synthesized *in vitro*, extracted with phenol, treated with alkali, and finally dissolved in 10 mM Tris·HCl, pH 7.5–0.1 M NaCl–0.1 mM EDTA (10). Hybridization assays contained 7.5 ng (10,000 ³H cpm) of either cDNA, and 1.2 μg of unlabeled, sheared and denatured virion DNA. Reassociation of 1.2 μg of virion DNA (10,000 ³H cpm) was monitored in a reference assay (●). Nucleic acids were incubated at 60° in 0.14 M sodium phosphate, pH 6.8–0.1% (w/v) $NaDodSO_4$ in volumes of 1 ml. At various times of incubation, 0.1-ml aliquots were removed and chromatographed on hydroxyapatite columns (11) to obtain C_0t values. A background of 5% of the radioactivity of denatured DNA eluted like double-stranded DNA from hydroxyapatite. No self-annealing of cDNA occurred under the conditions of this experiment.

Methods). RNA·DNA hybrids were denatured, and ad_2 mRNA was separated from the DNA fragments by sucrose gradient centrifugation (Fig. 2*b*). The precautions applied during preparative hybridization were not entirely successful. Whereas the starting material displayed the expected size profile (8, 9), with two peaks between 28S and 18S ribosomal RNA (Fig. 2*a*), the size distribution of RNA eluted from hy-

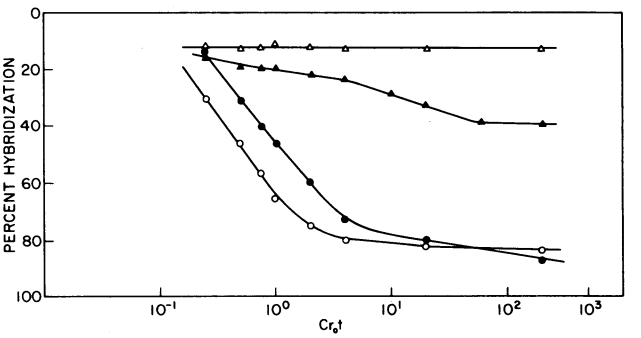

FIG. 4. Kinetics of hybrid formation between polysomal RNA and cDNA. Polysomal RNA extracted from ad_2-infected cells was purified either by oligo(dT)–cellulose chromatography (*closed symbols*) or by oligo(dT)–cellulose chromatography followed by preparative hybridization with virion DNA (*open symbols*). cDNA complementary to either RNA was synthesized *in vitro* (see *legend* of Fig. 3). Hybridization assays contained 7.5 ng (10,000 ³H cpm) of cDNA, and 13 μg of unlabeled, polysomal RNA, purified on oligo(dT)–cellulose, derived from either mock-infected (△,▲) or ad_2-infected (○, ●) KB cells. Hybrid formation was monitored as indicated in the *legend* of Fig. 3, and the initial concentration of RNA (A_{260} units) was multiplied by the time of incubation (hours) to obtain Cr_0t values.

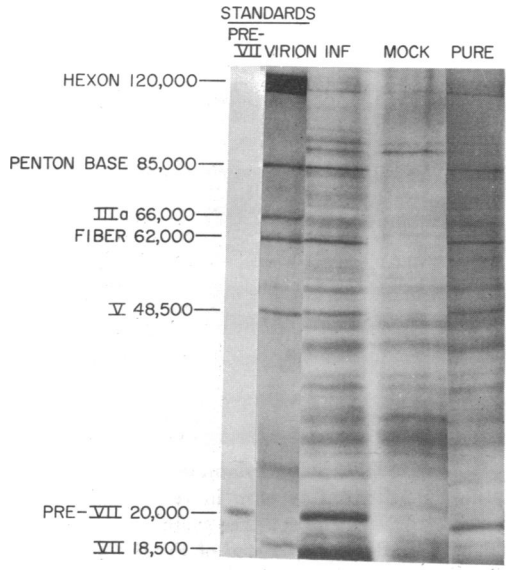

FIG. 5. NaDodSO$_4$–polyacrylamide gel electrophoresis of polypeptides synthesized *in vitro*. The cell-free system was programmed by oligo(dT)–cellulose purified polysomal RNA, derived from mock-infected (*MOCK*) or from ad$_2$-infected (*INF*) KB cells. In a separate assay, INF RNA was translated that had been further purified by preparative hybridization with ad$_2$ DNA (*PURE*). [^{35}S]Methionine-labeled marker proteins were derived from intact virions. Pre-VII, the precursor to protein VII (12), was purified from infected cells by NaDodSO$_4$–polyacrylamide gel electrophoresis (2). Molecular weights for virion polypeptides are cited from published work (12). The gel was autoradiographed for 3 days.

brids (Fig. 2*b*) indicated that annealing had caused some degradation, especially of large RNA molecules. As will be shown below, this degradation did not impede cell-free translation.

Both the partially purified RNA and the material purified further by preparative hybridization were transcribed *in vitro* into cDNA (10). Assuming nonselective transcription, all template RNA sequences, including unlabeled host cell RNA contaminants, should be represented by cDNA, which could then be used as a tool to determine the purity of ad$_2$ mRNA. Synthetic [^{3}H]cDNA transcribed either from RNA purified on oligo(dT)–cellulose or from RNA that had been further purified by preparative hybridization, was annealed with unlabeled virion DNA. Only half of the former cDNA annealed in the presence of excess amounts of ad$_2$ DNA. In contrast, re-

TABLE 1. *Exhaustive hybridization of mRNA*

DNA	Input cpm	Cpm of RNA hybridized
ad$_2$	10,000	8550
T7	10,000	410

Sonicated, heat-denatured DNA (20 μg) was hybridized with 1 μg of mRNA for 2 hr at 67° in 1 ml of 0.3 M NaCl–0.03 M sodium citrate, pH 7.0. Another 20 μg of DNA was added, and the mixture was incubated another 2 hr. The reaction was treated with 10 μg of RNase A (Worthington, boiled 10 min to destroy DNase activity) for 1 hr at 25°. The hybrids were precipitated on Millipore filters with cold trichloroacetic acid, and counted in a toluene-based liquid scintillation mixture.

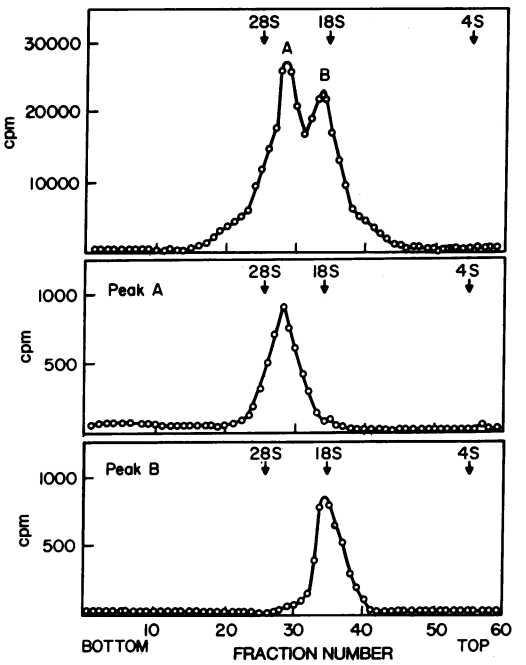

FIG. 6. Separation by size of partially purified ad$_2$ mRNA on sucrose gradients. RNA (100 μg), adsorbed on oligo(dT)–cellulose, was sedimented through a linear gradient of 15–30% (w/v) sucrose in 10 mM Tris·HCl, pH 7.5–0.1 M NaCl–0.1 mM EDTA. Centrifugation was for 3 hr at 4° at 55,000 rpm in the Spinco SW56 rotor. Fractions were collected from the bottom of the centrifuge tube, and radioactivity was determined by counting aliquots in Aquasol (*top panel*). $S_{20,w}$ values refer to [^{14}C]rRNA included as an internal size marker. Fractions 18–30 and 32–40, constituting pools of the two peaks (*A* and *B*) of RNA, were precipitated with ethanol, collected by centrifugation, dissolved in 1 mM potassium cacodylate pH 5.5–10 mM KCl, and sedimented through dimethylsulfoxide–sucrose gradients (8) for 18 hr at 25° at 55,000 rpm in the Spinco SW56 rotor. Fractions were collected, and aliquots were counted as above (*lower panels*).

association kinetics of unlabeled virion DNA and [^{3}H]cDNA, complementary to highly purified RNA, were indistinguishable from those of virion DNA itself (Fig. 3). Moreover, whereas part of the cDNA, complementary to RNA purified on oligo(dT)–cellulose, from infected cells could be annealed with RNA from mock-infected cells, transcripts of highly purified ad$_2$ mRNA would only anneal with RNA from infected cells (Fig. 4). These experiments clearly demonstrated that polysomal RNA from infected cells, purified by oligo(dT)–cellulose chromatography, contained host cell RNA sequences that could be removed by preparative hybridization of the RNA with virion DNA.

Cell-Free Translation of ad$_2$ mRNA. Both the ad$_2$ mRNA adsorbed on oligo(dT)–cellulose and the material further purified by preparative hybridization proved to be highly active messengers in a cell-free translation system derived from murine Krebs II ascites cells. With either RNA as template, polypeptides were produced *in vitro* that migrated with virion polypeptides during NaDodSO$_4$–polyacrylamide gel electrophoresis (Fig. 5). Proof of the identity between polypeptides synthesized *in vivo* and *in vitro* is given elsewhere (1, 2).

We decided to examine the coding capacity of each of the two size classes of ad$_2$ mRNA (see Fig. 2*a*). Fractions of each peak were pooled, and recentrifuged in the presence of di-

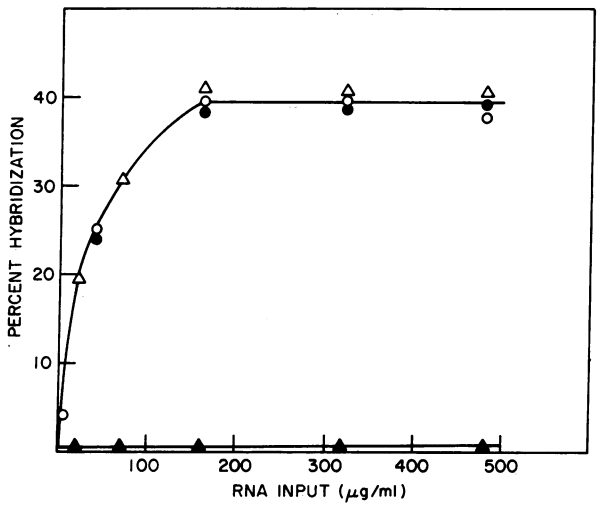

FIG. 7. Determination of the genetic complexity of ad_2 mRNA. RNA, purified on oligo(dT)–cellulose, was derived from polysomes of ad_2-infected cells. Increasing quantities of bulk RNA, and of each of the size-separated pools A and B (see Fig. 6) were hybridized with 12 ng of ad_2 [^{3}H]DNA that had been previously sheared and denatured. The nucleic acids were incubated in 0.1-ml hybridization assay mixtures (see *legend* of Fig. 3 for details) at 60° for 1 hr. RNA·DNA hybrids were adsorbed to hydroxyapatite (11), eluted with 0.4 M sodium phosphate, pH 6.8, and treated with 0.1 N NaOH for 18 hr at 37°, to hydrolyze more than 99.9% of the RNA. The amount of ad_2 [^{3}H]-DNA in each fraction was assayed by trichloroacetic acid precipitation onto Millipore filters, followed by counting in a toluene-based scintillation fluid.

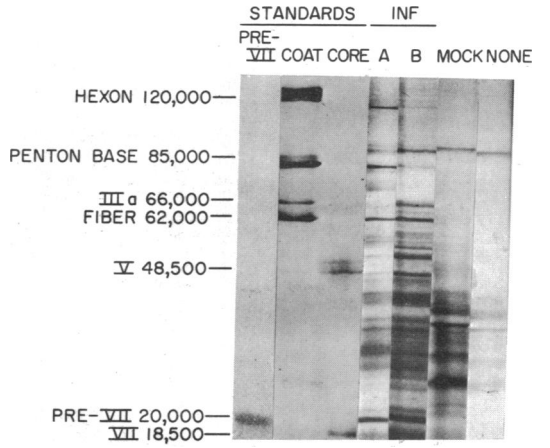

FIG. 8. NaDodSO$_4$-polyacrylamide gel electrophoresis of polypeptides synthesized *in vitro* in response to different size classes of ad_2 mRNA. The cell-free system was programmed by INF and MOCK RNA (see *legend* of Fig. 5 for definition), and by INF RNA that had been fractionated into classes A and B (see Fig. 6). Products of an endogenous synthesis without added mRNA are termed *NONE*. Virion marker proteins were prepared as indicated in Fig. 6. The gel was autoradiographed for 3 days.

methylsulfoxide to verify both the size separation and the integrity of the RNA (Fig. 6). No difference could be observed between the genetic complexities of the bulk RNA and those of the two separated size classes of RNA. Transcripts of 40% of the viral genome were represented in each case (Fig. 7). No complementarity could be detected between individual sequences of bulk RNA, i.e., all RNA was RNase A-sensitive after self-hybridization. We concluded that common RNA sequences were present in the separated size classes of RNA. Nevertheless, translation of RNA of either size *in vitro* revealed distinct patterns of polypeptides (Fig. 8). The larger RNA coded for hexon and penton base, the smaller RNA for polypeptide IIIa and V. Fiber and pre-VII, the precursor to VII (12), were synthesized predominantly by RNA species in the 22S area of the gradient (Eron, unpublished results), and thus appeared to be translated from RNA contained in each mRNA pool.

DISCUSSION

Adenovirus type 2 messenger RNA prepared by hybridization with virion DNA programs the synthesis of virion coat and core polypeptides in a cell-free system derived from Krebs II ascites tumor cells. The purity of mRNA was monitored with cDNA transcribed *in vitro* from RNA templates. The data demonstrate that RNA, purified on oligo(dT)–cellulose, extracted from polysomes of pulse-labeled, ad_2-infected cells contains considerable amounts of unlabeled host cell RNA. Some of this may represent ribosomal RNA not fully removed by oligo(dT)–cellulose chromatography. In addition, host cell mRNA synthesized before the pulse, and, therefore, not radioactive, may also contaminate the preparation (7).

Preparative hybridization of RNA purified on oligo(dT)–cellulose removes host cell sequences. This is seen by the fact that cDNA, synthesized from RNA eluted from viral DNA·RNA hybrids, anneals solely with viral sequences and does not hybridize with excess amounts of host cell RNA. However, the possibility remains that traces of biologically active host cell mRNA may fail to be transcribed into cDNA. If so, they are not detected by this procedure. Barring this unlikely event, we can state that the purification procedure described in this study yields pure ad_2 mRNA. We conclude that the polypeptides synthesized *in vitro* in response to this RNA are virus gene products.

The procedure outlined in this study of decoding pure ad_2 mRNA may now be extended to early RNA. Candidates for early virus proteins include enzymes and other proteins involved in nucleic acid synthesis, viral antigens, and viral gene functions responsible for malignant cell transformation. In a second application of this method, ad_2 mRNA may be hybridized with specific ad_2 DNA fragments (13), thereby separating classes of mRNA with unique primary structure. Cell-free translation of these populations of viral mRNA would represent a novel means of establishing a genetic map of virus gene functions.

Fractionation by size, as demonstrated previously (8, 9), yields distinct subpopulations of ad_2 mRNA. We find that each of the two discrete size classes of RNA has the same genetic complexity as the bulk RNA. This finding suggests that both size classes contain overlapping sequences of mRNA representing different stages of processing inside the infected cell. Nevertheless, size separation clearly resolves RNA molecules of different coding specificities. This may be due to true size differences among mRNA molecules specifying the various virus polypeptides. Alternatively, translation patterns may be influenced by processing and degradation of mRNA. The apparent large size of RNA molecules coding for some of the small virus polypeptides suggests that untranslated sequences are covalently attached to the message and/or that more than one polypeptide may be encoded in one mRNA molecule.

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1. Eron, L., Callahan, R. & Westphal, H. (1974) *J. Biol. Chem.* in press.
2. Eron, L., Westphal, H. & Callahan, R. (1974) *J. Virol.*, in press.
3. Aviv, H. & Leder, P. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 1408–1412.
4. Pettersson, U. & Sambrook, J. (1973) *J. Mol. Biol.* **73**, 125–130.
5. Kourilsky, P., Manteuil, S., Zamansky, M. H. & Gros, F. (1970) *Biochem. Biophys. Res. Commun.* **41**, 1080–1087.
6. Britten, R. J., Pavich, M. & Smith, J. (1970) *Carnegie Inst. Wash. Yearb.* **68**, 400–402.
7. Dinowitz, M. & Green, M. (1972) *Virology* **50**, 619–629.
8. Lindberg, U., Persson, T. & Philipson, L. (1972) *J. Virol.* **10**, 909–919.
9. Bhaduri, S., Raskas, H. J. & Green, M. (1972) *J. Virol.* **10**, 1126–1129.
10. Ross, J., Aviv, H., Scolnick, E. & Leder, P. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 264–268.
11. Khoury, G. & Martin, M. (1972) *Nature New Biol.* **238**, 4–6.
12. Anderson, C. W., Baum, P. R. & Gesteland, R. (1973) *J. Virol.* **12**, 241–252.
13. Pettersson, U., Mulder, C., Delius, H. & Sharp, P. A. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 200–204.