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Isolation of DNA Strand-Specific Early Messenger RNA Species in Cells Infected by Human Adenovirus 2

(DNA·RNA hybridization/gel electrophoresis/cycloheximide)

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ABSTRACT **Hybridization to the separated light (L) and heavy (H) strands of adenovirus 2 DNA in 50% formamide at 37° was used to isolate undegraded virus-specific RNA molecules from the polyribosomes of cycloheximide-treated human KB cells early after infection with adenovirus 2. About 20% of polyribosomal RNA labeled with [^{3}H]uridine from 4 to 7 hr after infection was virus-specific. Twice as much labeled RNA was homologous to the L strand as to the H strand. Polyacrylamide gel electrophoresis of RNA selected with unfractionated adenovirus DNA resolved a major component of virus-specific RNA in the 19–20 S region of the gel and smaller amounts of viral RNA in two heterogeneous fractions migrating at 15–18 S and 21–26 S. Selection with individual DNA strands showed that the 19–20 S main size class of early mRNA consists of two homogeneous RNA species with slightly different mobilities, the transcripts from the L and H strand having molecular weights of 7.4 × 10^5 and 7.7 × 10^5, respectively. The 15–18 S RNA hybridized with the L strand and the 21–26 S RNA with the H strand.**

Only one of the two complementary DNA strands in any region of a duplex DNA molecule can be transcribed to a functional messenger RNA for protein synthesis. With some DNA viruses transcription occurs exclusively from one strand of the viral genome (1), while with others regions from both strands are transcribed (2, 3). The human adenoviruses, especially the well-studied adenovirus type 2, form excellent models for study of the regulation of transcription in mammalian cells. Of special interest is the transcription of early adenovirus 2 genes that occurs in the absence of protein synthesis and thus probably reflects the transcription controls of the host cell (4). Early after infection only a few viral genes are transcribed to stable viral mRNA species (5), and these include those adenovirus genes expressed in transformed cells (6). It would be of interest, therefore, to be able to isolate individual adenovirus mRNA molecules in an undegraded form for studies on structure, sequence, and translation. The use of the separated strands of adenovirus DNA facilitates resolution of individual viral mRNA species. Landgraf-Leurs and Green (7) developed a procedure for the preparative separation of the adenovirus type 2 DNA strands, and showed that both strands were transcribed early as well as late after infection (8). Viral RNA isolated by hybridization to DNA by the usual annealing conditions is thermally degraded and cannot be used for further characterization.

Abbreviations: L and H strand, light and heavy strands as defined by the relative buoyant density of the DNA strand·poly(U,G) complex; EDTA, ethylenediaminetetraacetate.

Although undegraded adenovirus-specific RNA can be isolated on a preparative scale on the basis of its content of poly(adenylic acid) (9), this approach is useful only for the isolation of late viral RNA species which contain relatively little cell mRNA. Molecular hybridization to viral DNA is required to separate early viral mRNA from the much larger quantities of cell-specific RNA. The addition of 50% formamide to the hybridization mixture permits the lowering of the annealing temperature to 37°; RNA remains intact under such conditions (10, 11), thus permitting the isolation of undegraded viral mRNA molecules (12, 13). In this report we describe the selection of early adenovirus-specific RNA species by annealing to adenovirus 2 H and L DNA strands (see *Abbreviations* for terminology). The major component of virus-specific RNA observed at 19–20 S was shown to consist of two major species of nearly the same molecular weight.

MATERIALS AND METHODS

Cell Cultures and Virus. A freshly cloned line of KB cells was grown in suspension culture and infected with adenovirus 2 (strain 38–2) as described (4, 14). Virus was purified as reported earlier, except that treatment with a fluorocarbon was omitted (15) and a two-step equilibrium centrifugation in CsCl gradients was utilized. Virus stocks consisted of virus purified in the above manner using sterile glassware and reagents.

Viral DNA Strands. The isolation of the viral DNA and the preparative separation of the complementary DNA strands were performed as outlined by Landgraf-Leurs and Green (7) using a poly(U,G) preparation with a ratio of U:G of 1:0.3, purchased from Schwarz Bioresearch, Inc., lot number 6901. Hydroxyapatite chromatography (16) was used instead of the equilibrium centrifugation in CsCl gradients to separate L and H strands from undesirable reannealed double-stranded DNA as follows. Self-annealed strand preparations were adjusted to 5 ml in 0.14 M sodium phosphate buffer (pH 6.7) containing 0.4% sodium dodecyl sulfate and incubated with 1.5 ml of hydroxyapatite suspension (Bio-Rad Lab., Richmond, Calif.) for 15 min at 60°. Double-stranded DNA binds under these conditions and was removed together with the hydroxyapatite by low speed centrifugation. The purified L or H DNA strands were precipitated from the supernatant fluid with 2.5 volumes of ethanol, and dialyzed against 150 mM NaCl, 15 mM sodium citrate (pH 7.4). DNA concentrations were estimated from the absorbance at 260 nm.

Cell Fractionation and Isolation of RNA. KB suspension cells were concentrated by centrifugation and infected with 100

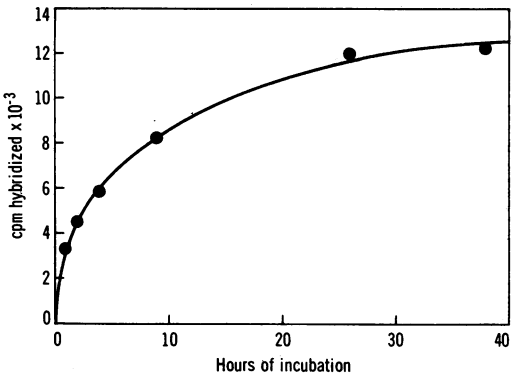

FIG. 1. Time course of hybridization in solution between adenovirus H strand DNA and polyribosomal RNA synthesized early after infection of cycloheximide-treated cells. Aliquots of early polyribosomal RNA (4.5 μg, 1.82 × 10^5 cpm) were annealed to 1 μg of H strand DNA in 0.8 ml of hybridization mixture. At the indicated times, the solution was processed to determine the amount of [^{3}H]RNA hybridized, as described in *Materials and Methods*.

plaque-forming units per cell of adenovirus 2 at a cell density of 3 × 10^6 cells per ml. After 1 hr for adsorption of virus, cells were diluted to 3.3 × 10^5 cells per ml with medium containing 25 μg of cycloheximide per ml (4). At 4 hr after infection, the cells in 500 ml of suspension culture were concentrated 10-fold in serum-free medium containing 25 μg of cycloheximide per ml, and labeled for 3 hr with 500 μCi/ml of [5,6-^{3}H]uridine (42.2 Ci/mmol, New England Nuclear Corp.). Cells were diluted tenfold with chilled phosphate buffered saline (PBS) lacking Ca^{++} and Mg^{++} (17) and harvested by centrifugation at 0°. In order to prepare polyribosomes, the cells were washed in ice-cold isotonic high pH (Iso-hi-pH) buffer (18) and lysed by the addition of 10 volumes of Iso-hi-pH buffer containing 0.5% Nonidet P-40 (Shell Chemical Corp.) and 40 μg/ml of dextran sulfate (Sigma Chemical Corp.) at 0°. After removal of nuclei by centrifugation at 800 × *g* for 3 min, and removal of the mitochondria by centrifugation at 12,000 × *g* for 10 min, the cytoplasmic supernatant was layered over 5 ml of a 1 M sucrose cushion in Iso-hi-pH buffer containing 100 μg/ml of dextran sulfate, and centrifuged in a Spinco Ti-50 rotor at 45,000 rpm for 2.5 hr at 0°.

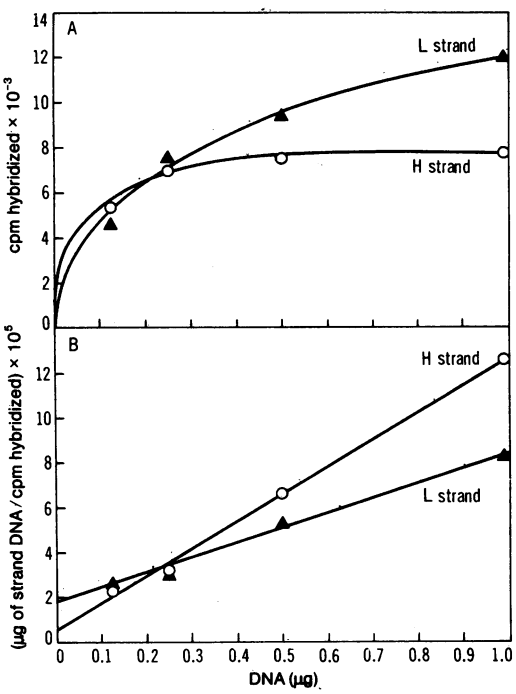

FIG. 2. Content of H and L strand-specific polyribosomal RNA early after infection with adenovirus 2. Increasing amounts of adenovirus H or L strand DNA were hybridized in solution to a constant amount of [^{3}H]uridine-labeled polyribosomal RNA (3.2 μg, 1.3 × 10^5 cpm), isolated early after infection, as described in the *legend* to Fig. 1. *A* is a conventional plot and *B* a reciprocal plot of the data according to Bishop *et al.* (24). Determination of the slope of the reciprocal plot reveals that at infinite DNA concentration, 8333 cpm or 6.4% of the input RNA would hybridize to H strand DNA and 17,857 cpm, or 13.7% of the input RNA to the L strand DNA. O: cpm of early polyribosomal [^{3}H]RNA hybridized to the H strand; ▲: cpm of early polyribosomal [^{3}H]RNA hybridized to the L strand.

The transparent polyribosomal pellet was suspended in 0.1 M Tris·HCl (pH 7.2), 0.02 M ethylenediaminetetraacetate (EDTA), 0.5% sodium dodecyl sulfate at 20° and extracted twice at 4° with a chloroform–phenol mixture [redistilled phenol saturated with 0.1 M Tris·HCl (pH 7.2), 2 mM EDTA, mixed with an equal volume of chloroform containing 1% isoamyl alcohol] (19). RNA was precipitated from the aqueous phase by adding NaCl to 0.17 M and 2.5 volumes of 95% ethanol at −20°.

DNA·RNA Hybridization. For the selection of the virus-specific RNA, 0.1 ml of polyribosomal RNA was annealed to 1 or 2 μg of H or L strand DNA (7) immobilized on 6.5-mm Schleicher and Schuell B-6 membrane filter discs (20). Hybridization was performed in 0.75 M NaCl, 0.1 M Tris·HCl (pH 7.4), 2 mM EDTA 0.5% sodium dodecyl sulfate containing 50% formamide (purified by crystallization and ether extraction) at 37° for 16 hr. Filter discs were rinsed five times with 0.1 M Tris·HCl (pH 8.0), 2 mM EDTA, 0.5% sodium dodecyl sulfate, incubated for 1 hr in fresh hybridization solution at 37°, and rinsed again with Tris–EDTA solution. No RNase treatment was employed. Hybridized RNA was eluted by incubating filters in 0.2 ml of 2 mM EDTA (pH 7.2) at 85° for 3 min. This procedure yielded virus-specific RNA with very little contamination from cell RNA. In control experiments in which labeled polyribosomal RNA from uninfected cells was annealed to adenovirus DNA, only 0.02–0.56% labeled RNA was recovered in the final elution as compared to infected cell RNA. To test the ability of RNA isolated in this manner to rehybridize to viral DNA strands, we used DNA·RNA hybridization in solution (21). The hybridization conditions were identical to those used for preparative hybridization on tilters except that treatment with RNase A (25 μg per ml) in 0.3 M NaCl, 0.03 M sodium citrate, pH 7.4 (2 × 55 C) for 1 hr at room temperature was included.

Polyacrylamide Gel Electrophoresis of Virus-Specific RNA. The size classes of purified virus-specific RNA were determined by the procedure of Loening (22) modified to include 0.5% agarose in the gel matrix, as suggested by Peacock and Dingman (23), and containing 0.2% Sarkosyl (Geigy Chemical Corp.) in the electrophoresis buffer. The 0.6-cm × 20-cm gels were prepared in glass tubes treated with Siliclad (Clay Adams) and subjected to electrophoresis for 4 hr at a constant 160 V in a water-jacketed apparatus at 20° with recirculation of the buffer.

The gels were fractionated in 2-mm portions with a commercial gel fractionator (Gilson Medical Electronics, Inc.) and counted in Aquasol (New England Nuclear Corp.) using a liquid scintillation spectrometer. ^{32}P-labeled ribosomal RNAs from KB cells served as markers.

RESULTS

Amount of H and L Strand-Specific RNA Associated with Polyribosomes Early after Adenovirus Type 2 Infection. The experiments analyzing the transcription of early adenovirus 2 genes were performed with cells exposed to cycloheximide at the end of the 1-hr adsorption period. The inhibitor of protein synthesis was used for two reasons. (*i*) The drug prevented the replication of adenovirus DNA, thereby suppressing the expression of late viral genes; and (*ii*) the amount of virus-specific RNA in polyribosomes early after infection is 5-fold higher in cycloheximide-treated cells than in untreated cells (4). We used DNA·RNA hybridization in solution to quantitate viral RNA, since it is more rapid and efficient than hybridization on filters. Hybridizations were conducted in 50% formamide at 37° as described in *Materials and Methods.*

Fig. 1 illustrates the time dependence of hybridization in solution between 1 μg of H strand DNA and early polyribosomal RNA from cycloheximide-treated cells labeled from 4 to 7 hr after infection. About one-half of the hybridizable RNA molecules had reacted by 5 hr. Hybridization was complete by 24 hr, with no increase occurring after 36 hr of incubation. At this time 6.6% of the input RNA had hybridized to the H strand. A time of 36 hr was routinely used therefore, for hybridization in solution.

Fig. 2 represents a typical saturation curve of polyribosomal RNA, labeled for 3 hr early after infection, with adenovirus 2 H and L DNA strands. Fig. 2*A* is a conventional plot and Fig. 2*B* represents a reciprocal plot of the data according to Bishop *et al.* (24). Graphic analysis of the latter reveals that 6.4% of the input RNA was homologous to the H strand and 13.7% of the input RNA was homologous to the L strand. We conclude that virus-specific RNA transcribed from the L strand is present in the polyribosomes early after infection at twice the concentration of the viral RNA that is transcribed from the complementary H strand.

Selection of H and L Strand-Specific Viral RNA. The technical details of the procedure used for the preparative selection of undegraded virus-specific RNA by hybridization to the individual adenovirus DNA strands and subsequent thermal elution are described in *Materials and Methods.* The use of 50% formamide in the hybridization mixture permitted the isolation of intact viral RNA molecules which were essentially uncontaminated by host cell RNA, even though the use of pancreatic RNase had been avoided. The strand-specificity of viral RNA isolated on individual DNA strands was tested by rehybridization to the homologous and heterologous strand in solution. Labeled polyribosomal RNA, isolated from cycloheximide-treated KB cells early after infection, was hybridized for 16 hr to filters containing H and L strand DNA. The hybridized RNA was eluted from the filters and rehybridized in solution to L or H DNA strands. About 70% of the strand-specific RNA rehybridized to the strand from which it originated. Only 7% of the RNA appeared to hybridize to the complementary strand (Table 1). Identical results were obtained when the RNA was hybridized and eluted twice before it was analyzed. These findings indicate that viral RNA eluted from the filters containing immobilized strand-specific DNA was at least 90% homologous to the original DNA strand. It is unclear, as yet, whether the apparent 10% cross-hybridization is indicative of base sequences common to both strands, or more likely was caused by a small amount of strand-specific DNA that was eluted from filters during elution of RNA, and annealed to its homologous RNA during rehybridization in solution, thus falsely scoring as hybrid.

TABLE 1. *Strand specificity of early polyribosomal RNA selected with H and L DNA strands*

Early viral [^{3}H]RNA		Rehybridization to 1 μg of	
		H strand	L strand
Eluted from	Input cpm	cpm bound (%)	
H strand DNA	4680	3220 (69)	333 (7)
L strand DNA	6880	613 (9)	5033 (73)

Virus-specific RNA was isolated by hybridization to H or L strand DNA immobilized on membrane filters. After thermal elution, strand-specific RNA was rehybridized in solution to each DNA strand.

Polyacrylamide Gel Electrophoresis of the H and L DNA Strand-Specific Early Virus-Specific RNA. The experiments described indicate that H and L strand-specific viral RNA was isolated under mild conditions which minimized contamination with host cell mRNA. In order to determine the size of the virus-specific RNA molecules, we performed polyacrylamide gel electrophoresis as described under *Materials and Methods.* Fig. 3*A* shows the profile of virus-specific RNA present in the polyribosomes early after infection, isolated by hybridization to unfractionated adenovirus 2 DNA. One predominant peak was found in the 19–20S region of the gel; much less virus-specific RNA migrated between 21 and 26S, and still less between 15 and 18 S. However, selection of virus-specific RNA by hybridization to H and L strand DNA revealed that the predominant component of early virus-specific RNA was in fact composed of two species of RNA with almost the same mobility. The H strand-specific RNA (Fig. 3*B*) was slightly larger than the L strand-specific RNA (Fig. 3*C*). Both major viral RNA peaks were well defined and nearly as sharp as the peak of the ribosomal 18S marker RNA, and thus could represent homogeneous messenger RNA species. The graphically determined molecular weights (23) were 7.7×10^5 for the major H strand-specific and 7.4×10^5 for the major L strand-specific RNA species. These differences were highly significant and were reproducibly obtained with five different preparations that were analyzed. There was also a difference in the distribution of the less conspicuous strand-specific viral RNA species. The RNA molecules larger than 20 S were mostly H strand-specific, while the RNA molecules smaller than 19 S were predominantly L strand-specific.

DISCUSSION

Before discussion of our results, two reports concerning the action of cycloheximide on RNA metabolism should be mentioned. Willems *et al.* (25) found that this inhibitor of protein synthesis suppressed the production of the mammalian 45S ribosomal precursor RNA. Gurgo *et al.* (26) reported that various drugs that inhibit protein synthesis produced a several-fold increase in the amount of messenger RNA as-

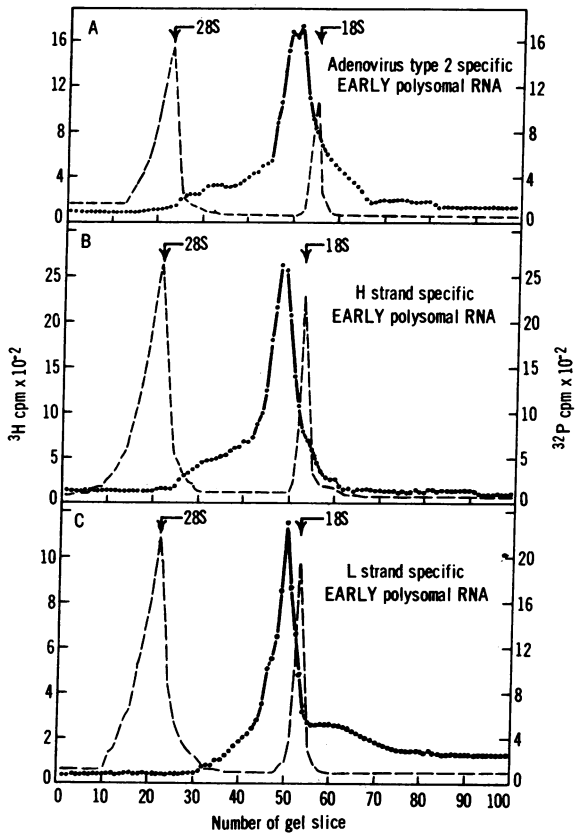

FIG. 3. Polyacrylamide gel electrophoresis of DNA strand-specific early viral RNA molecules. Early polyribosomal RNA labeled with [^{3}H]uridine was annealed to membrane filters containing 20 μg of denatured adenovirus 2 DNA(*A*), 2 μg of H strand DNA(*B*), or 2 μg of L strand DNA(*C*). Hybridized viral RNA (about 2% of input RNA) was eluted and subjected to electrophoresis on agarose–polyacrylamide gels as described in *Materials and Methods*. Ribosomal [^{32}P]RNA served as molecular weight markers (*arrows*). ●——●: cpm of virus-specific [^{3}H]RNA; – – –: cpm of ribosomal marker [^{32}P]RNA.

sociated with polyribosomes in prokaryotic cells. These effects of protein synthesis inhibitors may explain the five-fold increase in the amount of early adenovirus-specific RNA observed by Parsons and Green (4) in cycloheximide-treated cells. By DNA·RNA hybridization competition experiments, these authors showed that all early viral RNA sequences were transcribed in the presence of this drug. These results do not rule out, however, a quantitative alteration in the distribution of individual early viral RNA species due to a disturbance in viral RNA processing. This criticism also holds for the profiles of viral mRNA described in the present paper.

We observed that large quantities of two major viral mRNA species of very similar molecular weights were present in polyribosomes; one was transcribed from the H strand and the other from the L strand. In addition, much smaller quantities of H strand-specific RNA larger than the main species, and L strand-specific RNA smaller than the main species, were detected. Using DNA·RNA hybridization in solution, we determined that 20.1% of 3 hr-labeled polyribosomal RNA, isolated from cycloheximide-treated cells, was virus-specific. Of the total virus-specific RNA, 32% was homologous to the H strand, and 68% was homologous to the L strand. This agrees with the findings of Landgraf-Leurs and Green (8) that both DNA strands are transcribed early in adenovirus 2 infection. The fact that twice as much viral RNA associated with polyribosomes early after infection is transcribed from the L strand as from the H strand leads to an interesting problem in the control of transcription. Two types of control may be envisioned: (*i*) the more rapid synthesis of L strand-specific RNA, or (*ii*) the more rapid degradation of H strand-specific RNA. A third possibility is that the increased amount of L strand RNA is an artifact of the cycloheximide treatment.

Control experiments provide good evidence that the "selected RNA" in our experiments was uncontaminated by host-cell RNA, and that it was strand-specific. The remarkable homogeneity of the major peaks of RNA selected by L and H strands, as compared to ribosomal RNA, is strong evidence for lack of RNA degradation. The electrophoretic distribution of the early virus-specific RNA species (Fig. 3) selected on unfractionated viral DNA is compatible with the results obtained by other authors (4, 9) using "unselected RNA", i.e., RNA fractionated on gels and virus-specific species identified by hybridization of gel fractions with viral DNA. Our major RNA fraction of 19–20 S probably corresponds to component I of Parsons and Green (4), and the E_3 RNA species of Lindberg *et al.* (9). Both groups also observed less well-defined virus-specific RNA smaller than the main fraction (component II of Parsons and Green and the RNA species E_1 and E_2 of Lindberg *et al.*) and broadly distributed virus-specific RNA that was larger than the main fraction [component III of Parsons and Green (4) and the "heterogeneous population" of viral RNA of Lindberg *et al.* (9)]. The major portion of our "selected" viral RNA is present in the main 19–20S RNA fraction, while studies using unselected RNA (4, 9) found somewhat higher relative proportions of the smaller and larger viral RNA species. These differences may be related to hybridization procedure, i.e., the preparative isolation of viral RNA and its subsequent display on gels as reported here, as compared to electrophoresis first and identification by hybridization of each fraction, as reported by others (4, 9).

The broad distribution of the minor higher-molecular-weight RNA homologous to the H strand and the minor lower-molecular-weight RNA homologous to the L strand contrasts with the homogeneity of the two major RNA species. It is possible that these minor components represent an unresolved mixture of several virus-specific RNA species. Another possibility is that they represent, or are contaminated with, intermediates in the processing of virus-specific RNA that leak from the nucleus or accumulate in the presence of cycloheximide, and cosediment with or bind to polyribosomes. This possibility is consistent with the report by Wall *et al.* (18) of high-molecular-weight virus-specific RNA species in the nucleus early after infection, and recent hybridization data by Green *et al.* (27) showing that transcripts from about 40% of the L and 55% of the H strand occur early after infection, but that only 14 and 14% represent stable RNA species.

In a study on virus-specific RNA isolated from rat embryo cells transformed by adenovirus 2, Wall *et al.* (28) observed three components of virus-specific RNA associated with polyribosomal RNA, with two minor components sedimenting at about 16 and 26 S and one major component at 20 S. This distribution resembles our profile of the early viral RNA species selected on unfractionated adenovirus 2 DNA. Fujinaga and

Green (6) have shown by hybridization-competition experiments that only a subset of the genes transcribed early during productive infection is expressed in rat embryo cells transformed by adenovirus 2. One might, therefore, speculate that the major 19–20S RNA species present in the polyribosomes of transformed cells is transcribed from only one strand of the viral genome.

Because of the relatively small amounts of genetic information transcribed early during adenovirus 2 infection, the selection of strand-specific RNA could be used to isolate viral mRNA for several important uses, including the physical mapping of the early adenovirus genes by DNA·RNA hybridization and visualization in the electron microscope, and the translation of viral mRNA in cell-free protein-synthesizing systems or in amphibian oocytes. Only a small number of early viral proteins is expected, and among these may be proteins responsible for the maintenance of the transformed cell. Of interest in this connection is the synthesis of two early proteins of molecular weight 75,000 and 45,000 (29) which are associated with a DNA replication complex isolated from adenovirus 2 infected KB cells. Proteins of similar molecular weights which bind to single-stranded DNA were reported to be present in monkey kidney cells abortively infected with adenovirus 5 (30). These are of the appropriate size for coding by the major viral mRNA of molecular weight 7.4 or 7.7 $\times$ 10^5, and the smaller species sedimenting at 15–18 S.

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