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In Vitro Translation of Adenovirus Type 12-Specific mRNA Isolated from Infected and Transformed Cells

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The early and late gene products of human adenovirus type 12 (Ad12), as well as the viral proteins synthesized in an Ad12-transformed cell line, were identified by translation of viral mRNA in an in vitro protein-synthesizing system. Cytoplasmic RNA was isolated from permissive KB or nonpermissive BHK cells infected with Ad12 and from Ad12-transformed HA12/7 cells. Virus-specific RNA was selected by hybridization to Ad12 DNA covalently bound to cellulose. Viral RNA was then translated in a fractionated rabbit reticulocyte cell-free system or in wheat germ S-30 extracts. The proteins synthesized were characterized by immunoprecipitation and subsequent electrophoresis on sodium dodecyl sulfate-polyacrylamide gels. RNA prepared from KB cells late after infection with Ad12 elicited the synthesis of most of the structural polypeptides of the virion and at least two presumably nonstructural Ad12 proteins. When viral RNA isolated early after infection of KB cells with Ad12 was translated in vitro, 10 polypeptides were observed: E-68K, E-50K, E-42K, E-39K, E-34K, E-21K, E-19K, E-13K, E-12K, and E-10K. Ad12-specific RNA was also isolated from the Ad12-transformed hamster cell line HA12/7, which contains several copies of the Ad12 genome integrated in the host genome. The RNA codes for at least seven polypeptides with molecular weights very similar to those of the early viral proteins.

The productive infection of human KB cells by adenovirus type 12 (Ad12) proceeds in at least two distinct phases. During the early period, which precedes the onset of viral DNA replication, only a limited portion of the viral genome is expressed (34). In the late phase, a large part of the genome is transcribed into functional viral RNA (34).

In baby hamster kidney (BHK-21) cells, however, Ad12 undergoes an abortive cycle (4, 36). Since viral DNA replication cannot be detected in these cells, the block, which prevents virus multiplication, must be located in a viral or cellular function required early in infection (4-6, 14). The mRNA's isolated from abortively infected BHK-21 cells are derived from the same or similar regions of the Ad12 genome as the mRNA's made early after productive infection (25, 26, 29). During abortive infection, a small portion of the cells becomes transformed. These cells do not produce virions or viral structural proteins; they do, however, contain T antigen (16, 18, 19). Four Ad12-transformed hamster cell lines have been characterized in this laboratory with respect to the transcription of viral DNA sequences into mRNA and the persistence and the physical state of the viral DNA (14, 15, 25, 26). In each of the cell lines tested, the viral genome persists in multiple copies in an integrated state (15, 37), and different fractions of the viral genome are represented in unequal amounts (14). However, only those portions of the Ad12 genome which are expressed early after productive infection are transcribed into mRNA in these cells (25, 26).

Some of the viral functions expressed early might play an important role in the initiation and maintenance of the transformed state. The identification of the viral gene products in transformed cells, as well as in Ad12-infected cells, is therefore of particular interest.

Cell-free systems capable of translating mammalian mRNA are useful tools for studying the expression of viral genes in host cells (1, 2, 12, 20, 21, 28, 30). Such systems provide one possible approach toward the identification of virus-coded proteins and their functions. For adenovirus type 2 (Ad2), approximately 19 virus-specific proteins have been found in extracts of infected cells, and at least 16 of these proteins have been identified among the products of cell-free translation of cytoplasmic viral RNA (2, 12, 20, 21). Apart from the Ad12 virion proteins, little is known at present about the Ad12 gene products and their functions. Among the early Ad12-specific proteins which have been charac-

terized recently are a tumor-specific antigen (18, 19) and a DNA-binding protein (32, 33).

In the present report, we describe experiments in which early and late gene products of Ad12 and viral proteins synthesized in one Ad12-transformed hamster cell line have been identified by in vitro translation of viral RNA isolated from infected or transformed cells. The products of the cell-free translation system were detected by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and characterized as virus-specific by immunoprecipitation.

MATERIALS AND METHODS

Cells and virus. Human KB cells (CCL17) were obtained from the American Type Culture Collection. BHK-21 cells were a gift of P. Faulkner, Hamilton, Ontario, Canada. The HA12/7 cell line was derived by H. zur Hausen from primary Syrian hamster kidney cells by transformation with Ad12 (39). KB cells were propagated in monolayers or suspension cultures in Eagle medium or in Eagle medium modified for suspension cultures (10) supplemented with 10% calf serum (Grand Island Biological Co. [GIBCO], Grand Island, N.Y.). BHK-21 and HA12/7 cells were grown in Dulbecco medium (3, 9) containing 10% fetal calf serum (Flow Laboratories, Inc., Rockville, Md.). Large quantities of HA12/7 cells were propagated in suspension cultures in Eagle medium containing 10% calf serum. Human Ad12 (a gift of W. Rowe, National Institutes of Health, Bethesda, Md.) was propagated in suspension cultures of KB cells. The purification of the virus has been described elsewhere (4).

Viral DNA. Ad12 DNA was extracted from CsCl-purified virions by the SDS-Pronase B-phenol method described previously (7). All Ad12 preparations used in this study were identified by the specific *Bam*HI restriction endonuclease pattern of the viral DNA extracted from these preparations (26).

Covalent linkage of viral DNA to cellulose. Single-stranded Ad12 DNA was covalently linked to aminobenzyloxymethylcellulose after diazotization of the primary acrylamino groups as described by Miles and Hales (23) and Noyes and Stark (24).

Aminocellulose was dissolved in ammoniacal $Cu(OH)_2$ [0.45 g of $Cu(OH)_2$, 0.1 g of sucrose, and 6 ml of NH_4OH (specific gravity, 0.88 g/ml) per 10 ml of solution], precipitated by dropwise addition of 10% H_2SO_4 to pH 6, and washed three times by suspension in ice-cold water before diazotization. Briefly, about 75 mg of precipitated cellulose was suspended in 8 ml of water at 0°C, and 16 ml of 1.8 M HCl and 0.64 ml of a freshly prepared solution of $NaNO_2$ (10 mg/ml in water) were added. The reaction mixture was stirred for 30 min at 0°C, and excess HNO_2 was destroyed by adding solid urea until reaction with starch-iodide paper was negative (23). The diazotized cellulose was collected by centrifugation, washed once each with cold water, cold 0.2 M borate (pH 8), and 80% dimethyl sulfoxide, and was subsequently resuspended in the DNA solution in 80% dimethyl sulfoxide at a cellulose concentration of about 20 mg (dry weight) per ml. The reaction mixture was incubated at 4°C with continuous stirring for 48 h. The DNA-cellulose was collected by centrifugation and washed at least twice in 80% dimethyl sulfoxide, four times in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at room temperature, and twice in 99% formamide–0.1% SDS at 80°C. The DNA-cellulose was then washed twice in hybridization buffer (50% formamide, 0.6 M NaCl, 0.1% SDS, 0.1 M Tris-hydrochloride, pH 7.4, 0.001 M EDTA) and stored at 4°C.

Before linkage to aminocellulose, the DNA was sheared to fragments of about 10 to 15S and denatured at pH 12 immediately before coupling.

Infection of cells with Ad12 and preparation of cytoplasmic RNA. Cell cultures were inoculated with Ad12 at multiplicities of infection of 50 to 100 PFU per cell. Cytoplasmic RNA from uninfected cells, infected cells, or Ad12-transformed cells was prepared as follows. Cells were collected by centrifugation and washed once with phosphate-buffered saline. The packed cells were suspended in about 10 volumes of ice-cold isotonic buffer (0.01 M Tris-hydrochloride, pH 7.4, 0.15 M NaCl, 0.002 M magnesium acetate) containing 0.5% Nonidet P-40 (Shell Co.) and 20 μg each of cycloheximide and Macaloid (National Lead Co., Houston, Tex.) per ml. After 5 to 10 min at 0°C, the nuclei were removed by centrifugation. The cytoplasmic extract was made 0.5% in SDS and 0.005 M in EDTA. The mixture was extracted three times with phenol-chloroform (1:1) previously saturated with 0.01 M Tris-hydrochloride (pH 7.6)–0.15 M NaCl–0.005 M EDTA. The RNA was precipitated twice with 2.5 volumes of ethanol at −20°C, resuspended in water, and stored at −20°C.

Selection of viral RNA by hybridization to Ad12 DNA. DNA-RNA hybridization experiments were carried out in a total volume of 1 to 2 ml of 50% formamide hybridization buffer containing 0.5 mg of *Escherichia coli* rRNA per ml. DNA-RNA hybridization assays contained about 150 μg of single-stranded Ad12 DNA covalently linked to 20 mg of diazotized cellulose and about 1 mg of cytoplasmic RNA which was prepared from uninfected, Ad12-infected, or Ad12-transformed cells. The DNA-RNA mixture was heated to 80°C for 2 min, cooled to 37°C, and hybridized for 24 to 36 h at 37°C. Upon hybridization, samples were placed on ice and centrifuged for 1 min to pellet the DNA–cellulose. The supernatant solution was removed carefully, and the cellulose was washed three times with 1 to 2 ml of 2× SSC and three to five times with 25% formamide–0.1 M Tris-hydrochloride (pH 7.4)–0.6 M NaCl–0.1% SDS. Ad12-specific RNA was eluted from the DNA–cellulose by resuspending the cellulose in 0.5 ml of 99% formamide–0.1% SDS and heating the mixture to 60°C for 1 min. The procedure was repeated once, and the RNA from the combined eluates was precipitated by 2.5 volumes of ethanol at −20°C in the presence of about 100 μg of rat liver or wheat germ tRNA per ml. The precipitate was resuspended in water and stored at −20°C.

Analysis of RNA by polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis in the presence of 98% formamide was carried out as described previously (8). Upper and lower buffers were 0.02 M sodium phosphate (pH 6.8) in 98% formamide. RNA samples in 0.2 ml of a mixture containing 84%

buffered formamide and glycerol were applied to 10-cm cylindrical gels consisting of 3.5% polyacrylamide in 98% buffered formamide and were subjected to electrophoresis for 16 h at 100 V and room temperature. After electrophoresis, the gels were cut into 1.5-mm-thick slices, and the RNA from each slice was eluted by dissolving the acrylamide slices in dimethyl sulfoxide.

Subsequently, the samples were counted in a scintillator of toluene-methanol (1:1), containing 2,5-diphenyloxazole and 2,2-*p*-phenylen-bis-(5-phenyloxazole).

Cell-free protein synthesis. (i) Rabbit reticulocyte cell-free system. The fractionated rabbit reticulocyte system of Schreier and Staehelin (35) was used with some modifications. To induce reticulocyte formation, rabbits were injected subcutaneously for 5 consecutive days with 0.3 ml of a 2.5% solution of phenylhydrazine per kg of body weight. Blood cells were collected by centrifugation for 10 min at 5,000 rpm in a Sorvall GSA rotor and washed three times by suspending the cells in 0.14 M NaCl-0.005 M KCl-0.005 M $MgCl_2$ and pelleting at 5,000 rpm for 10 min in a Sorvall SS34 rotor. The cells were lysed by suspending 1 volume of packed cells in 2 volumes of 0.004 M $MgCl_2$-0.001 M dithiothreitol (DTT). After 90 s, lysis was stopped by the addition of sucrose and KCl to final concentrations of 0.25 and 0.3 M, respectively. Postmitochondrial supernatant was prepared by centrifuging the lysate for 15 min at 30,000 × *g* in a Sorvall SS34 rotor. Subsequently, polysomes and ribosomes were sedimented for 3 h at 100,000 × *g* in a Beckman 75Ti rotor. After withdrawal of the high-speed supernatant, the ribosomal pellet was suspended in 0.25 M sucrose-0.005 M Tris-hydrochloride (pH 7.6)-0.001 M DTT-0.0001 M EDTA at a final concentration of 300 optical density units at 260 nm (OD_{260} units) per ml and quickly frozen in liquid nitrogen. Ribosomal subunits were prepared according to published procedures (13). First, polysomes were preincubated at 37°C to allow termination of previously initiated polypeptide chains. The complete incubation mixture contained the following per milliliter: 40 OD_{260} units of polysomes and ribosomes, 0.4 ml of the pH 5 enzymes (see below), 20 mM creatine phosphate, 40 μg of creatine phosphokinase, 1 mM ATP, 0.4 mM GTP, and 40 mmol of each of the 20 L-amino acids. The final ionic concentrations were 0.003 M magnesium acetate, 0.11 M KCl, 0.001 M DTT, and 0.03 M Tris-hydrochloride, pH 7.6. After incubation for 45 min at 30°C, the mixture was chilled, and KCl was added to a final concentration of 0.5 M.

The ribosomal subunits were separated on a linear 5 to 30% sucrose gradient containing 0.02 M Tris-hydrochloride, (pH 7.4), 0.003 M magnesium acetate, 0.3 M KCl, and 0.001 M DTT. Centrifugation was carried out in a Beckman SW27 rotor for 7 h at 24,000 rpm at 3°C. After the fractions were collected, the optical density profile of the gradient was measured at 260 nm. The 60S and 40S peak fractions were pelleted by centrifugation at 100,000 × *g* in a Beckman 75Ti rotor at 3°C. The purified 60S and 40S ribosomal subunits were suspended in 0.1 M NH_4Cl-0.003 M magnesium acetate-0.001 M DTT-0.02 M Tris-hydrochloride, pH 7.6, and mixed in a ratio of 2.5:1. The mixture was frozen in liquid nitrogen and stored at −80°C.

The pH 5 enzyme fraction was obtained by disrupting rat liver cells in a Dounce glass homogenizer and by centrifuging the extracts at 100,000 × *g* for 50 min. The pH was subsequently adjusted to 5.0 by adding 1 M acetic acid. The precipitate formed was pelleted and redissolved in 0.1 M NH_4Cl-0.003 M magnesium acetate-0.001 M DTT-0.001 M EDTA-0.02 M Tris-hydrochloride, pH 7.4.

Initiation factors were prepared from rabbit reticulocytes according to published procedures (2, 35). Crude polysomes and ribosomes were added at a concentration of 100 OD_{260} units per ml in 0.25 M sucrose-0.005 M Tris-hydrochloride (pH 7.6)-0.001 M DTT-0.0001 M EDTA. A 4 M KCl solution was subsequently added slowly with stirring to a final concentration of 0.5 M. Stirring was continued at 0°C for 15 min, and the ribosomes were then sedimented for 4 h at 100,000 × *g* in a Beckman 75Ti rotor at 3°C. The supernatant (designated ribosomal wash) was fractionated by $(NH_4)_2SO_4$ precipitation at 30 to 40% (fraction A) and 40 to 70% (fraction B). The precipitates were collected by centrifugation and dissolved in 0.02 M Tris-hydrochloride (pH 7.6)-0.001 M DTT-0.0002 M EDTA-0.12 M KCl. Fraction A was further purified on a DEAE-cellulose column equilibrated with 0.02 M Tris-hydrochloride (pH 7.6)-0.001 M DTT-0.0002 M EDTA-10% (vol/vol) glycerol-0.12 M KCl. The material that eluted from the column between 0.15 and 0.3 M KCl was used (fraction A_1).

The standard in vitro translation mixture (100 μl) contained about 0.6 OD_{260} unit of purified ribosomal subunits, 10 μl of pH 5 enzymes (110 μg of protein), 10 μg of fraction A_1 and 75 μg of fraction B of the initiation factors, about 8 μg of rat liver tRNA (prepared by phenol extraction and ethanol precipitation of a post-ribosomal supernatant [13]), 1.0 mM ATP, 0.4 mM GTP, 20 mM creatine phosphate, 4 μg of creatine phosphokinase, 30 mM HEPES (*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid; pH 7.6), 110 mM KCl, 3 mM magnesium acetate, 30 to 40 μM each of the unlabeled amino acids, 20 μCi of [^{35}S]methionine (specific activity, 770 Ci/mmol), and RNA as indicated. Reaction mixtures were incubated for 60 min at 37°C.

(ii) Wheat germ cell-free system. Wheat germ S-30 extracts were prepared by the procedure described by Roberts and Paterson (30) with the following modifications. After the centrifugation of the wheat germ homogenate at 30,000 × *g* for 15 min at 0°C, the supernatant was quickly frozen in liquid nitrogen. The S-30 extract was preincubated for 10 min at 30°C. Immediately before the extracts were used, they were subjected to gel filtration on a Sephadex G25 or Bio-Gel P20 column in 30 mM HEPES (pH 7.6)-120 mM KCl-3 mM magnesium acetate-3 mM β-mercaptoethanol in volumes of about 0.25 ml. For gel filtration, a modification of the microcentrifuge gel filtration method described by Emneus (11) was used. A standard protein synthesis assay contained in a final volume of 100 μl about 2.5 OD_{260} units of preincubated wheat germ S-30 extract, 30 mM HEPES

(pH 7.6), 2 mM DTT, 1 mM ATP, 0.2 mM GTP, 8 mM creatine phosphate, 4 μg of creatine phosphokinase, 30 to 40 μM unlabeled amino acids, 100 mM KCl, 3 mM magnesium acetate, 20 μCi of [^{35}S]-methionine (specific activity, 770 Ci/mmol), and RNA as indicated, as well as 7.5 μg of tRNA extracted from wheat germ by the method described by Roberts et al. (31). Reaction mixtures were incubated for 60 min at 30°C.

Preparation of antisera. The following types of antisera against Ad12 proteins were prepared: (i) antisera against Ad12 particle proteins were obtained from rabbits after three injections with highly purified Ad12 preparations; (ii) antisera against Ad12 proteins were prepared by using extracts obtained at 10 h postinfection of KB or BHK-21 cells with Ad12; (iii) antisera against the Ad12 proteins expressed in the Ad12-transformed cell line HA12/7 (39) were obtained from hamsters bearing tumors initiated by a subcutaneous injection of 10^6 HA12/7 cells; (iv) normal rabbit and hamster sera were drawn from nonimmunized animals.

All antisera were heated to 55°C for 10 min to inactivate complement and to reduce protease activity. Goat anti-rabbit immunoglobulin and rabbit anti-hamster serum were obtained from GIBCO.

Immunoprecipitation. Immunoprecipitation was performed by incubating 100 μl of the in vitro translation assay with 10 to 30 μl of the appropriate antiserum directed against Ad12 protein. After 30 min at 25°C, 0.1 ml of goat anti-rabbit or rabbit anti-hamster immunoglobulin was added at equivalence, and incubation was continued for 6 to 8 h at 4°C. The samples were then centrifuged at 5,000 × *g* for 10 min at 4°C. The pellets were washed twice with 0.5 ml of 10 mM sodium phosphate (pH 7.2)-150 mM NaCl-0.5% Nonidet P-40-0.5% deoxycholate and once with 0.5 ml of distilled water.

The pellets were finally solubilized in sample buffer (0.5 M Tris-hydrochloride, pH 8.8, 2% SDS, 0.14 M β-mercaptoethanol, 0.002 M EDTA, 10% glycerol), boiled for 5 min, and applied to the gel.

Identification of products. The in vitro products were analyzed by SDS-polyacrylamide gel electrophoresis as described previously (17, 37). The distribution of proteins in the gel was analyzed by autoradiography after drying the gels (22). Film used for autoradiograms was Kodak RP/S X-Omat or du Pont Cronex Z.

Estimates of molecular weights. The molecular weights of Ad12 particle proteins were determined by comparing them with the molecular weights of the Ad2 particle proteins and those of markers with known molecular weights (Boehringer Mannheim Corp. calibration kit; Fig. 1). Electrophoresis was performed in slab gels as described previously (37) containing 13% acrylamide and 1% SDS. After electrophoresis, the proteins were stained with 0.25% Coomassie brilliant blue in 5% methanol and 7.5% acetic acid, and the gels were dried under vacuum.

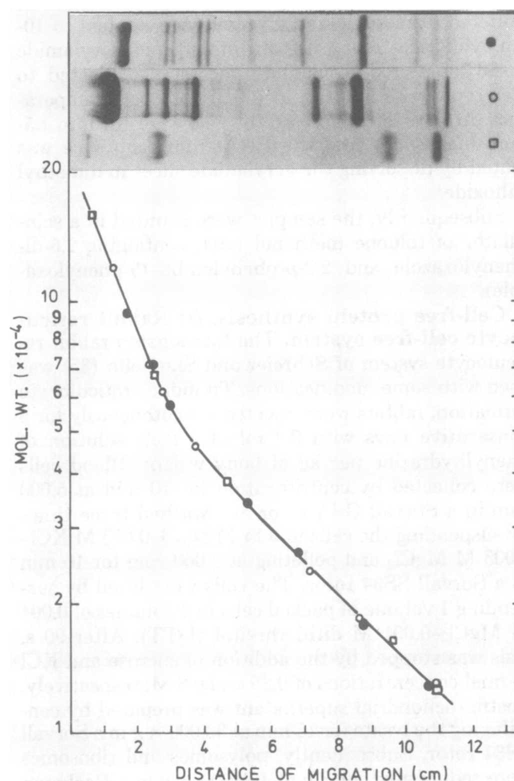

FIG. 1. *Molecular weight determination of Ad12 virion proteins. Molecular weights were plotted versus the electrophoretic mobility in 13% polyacrylamide gels containing SDS. Symbols: ○, Ad2 particle proteins (27); ●, Ad12 particle proteins; □, marker proteins of known molecular weight (β and β' subunits from E. coli RNA polymerase [160K molecular weight], bovine serum albumin [68K molecular weight], α subunit of the E. coli RNA polymerase [39K molecular weight], trypsin inhibitor from soybeans [21.5K molecular weight], cytochrome c [12.5K molecular weight]). Electrophoresis was for 7 h at a constant current (50 mA).*

RESULTS

Preparation of Ad12-specific mRNA. Nucleic acid hybridization techniques were used to select preparatively viral RNA from cytoplasmic RNA isolated from Ad12-infected cells. It was essential to minimize degradation of the RNA which was used for in vitro translation. We chose the liquid hybridization procedure of RNA to DNA which was covalently coupled to cellulose (see above).

About 0.5 to 0.7% of the labeled total cytoplasmic RNA prepared early postinfection of KB or BHK-21 cells with Ad12 and about 4 to 6% of the RNA prepared late from Ad12-infected KB cells hybridized specifically to Ad12 DNA (Table 1). These data agreed well with previous results (26). For 70 to 80% of the material binding to Ad12 DNA-cellulose, the hybridization was virus specific, as shown by the results of the

TABLE 1. *Selection of Ad12-specific RNA by hybridization to Ad12 DNA*[a]

Cell type	Time after infection (h) when RNA was prepared	Total cytoplasmic RNA				
		RNA used for hybridization	RNA hybridized in step 1		RNA from the first hybridization which was eluted and rehybridized to Ad12 DNA	
			cpm	%	cpm	%
KB	Uninfected	1.09×10^6	3.71×10^3	0.34	90×10^1	2.4
KB	9	2.58×10^6	1.83×10^4	0.71	1.29×10^4	70.8
KB	10	1.40×10^6	8.26×10^3	0.59	6.13×10^3	74.2
KB	30	2.01×10^6	1.13×10^5	5.6	8.97×10^4	79.4
KB	40	2.35×10^6	1.03×10^5	4.4	8.41×10^4	81.7
BHK-21	Uninfected	1.72×10^6	4.64×10^3	0.27	1.44×10^2	3.1
BHK-21	10	2.41×10^6	1.47×10^4	0.61	1.01×10^4	68.6
BHK-21	12	0.97×10^6	4.65×10^3	0.48	3.32×10^3	71.5
BHK-21	16	1.38×10^6	6.07×10^3	0.45	4.19×10^3	69.0
HA12/7		3.14×10^6	1.79×10^4	0.57	1.37×10^4	76.5
HA12/7		2.80×10^6	1.15×10^4	0.41	8.06×10^3	70.1

[a] Efficiency of selection of virus-specific RNA by hybridization of total cytoplasmic RNA to Ad12 DNA covalently linked to cellulose. The cytoplasmic RNA was prepared from Ad12-infected and uninfected KB and BHK-21 cells and from the Ad12-transformed cell line HA12/7 (39) as described in the text. The last two columns give data of experiments in which the RNA from the first hybridization was subjected to a second hybridization step. In all experiments, 150 μg of Ad12 DNA covalently bound to cellulose was used. Conditions of hybridization and elution of the RNA were described in the text.

second hybridization step (Table 1). To ascertain that during selection of viral RNA by hybridization to Ad12 DNA certain species of viral RNA were not lost, the preselected RNA from Ad12-infected cells was characterized by electrophoresis on polyacrylamide gels containing 98% formamide. The patterns of distribution of the RNA were very similar regardless of whether the RNA was preselected once (Fig. 2B) or twice (Fig. 2C) by hybridization to Ad12 DNA, but differed markedly from the pattern of unselected RNA (Fig. 2A). Thus, it appeared unlikely that specific size classes of viral RNA were lost during the selection procedure.

Characterization of the reticulocyte and wheat germ translation systems. Incorporation of amino acids into proteins in the fractionated reticulocyte and wheat germ cell-free systems was stimulated by the addition of unselected cytoplasmic RNA prepared late from Ad12-infected KB cells. Maximal protein synthesis was observed in both systems with 15 to 25 μg of RNA per 100 μl of reaction mixture. Most of the synthesis occurred during the first 45 to 60 min of incubation. The optimal Mg^{2+} and K^+ concentrations for the reticulocyte cell-free system were 3.2 mM and 100 to 110 mM, respectively. In all experiments subsequently described, optimal conditions were used.

Cell-free translation of late Ad12-specific RNA. Late viral RNA used to program cell-free synthesis was isolated 30, 38, or 46 h after infection of KB cells with Ad12. Total cytoplasmic RNA was isolated, and the viral RNA was selected by hybridization to Ad12 DNA as described above. Figure 3 shows the results of an analysis of the polypeptides synthesized in the reticulocyte system programmed with unselected or selected late Ad12 mRNA. When no RNA was added to the system, only a few faint polypeptide bands could be recognized (Fig. 3, lanes B and I). Synthesis programmed by cytoplasmic RNA from Ad12-infected cells produced a complex pattern of polypeptides (Fig. 3, lane C) resembling the pattern of the polypeptides synthesized in Ad12-infected cells in vivo (Fig. 3, lane D). Cytoplasmic RNA extracted from cells 30, 38, or 46 h postinfection (Fig. 3, lane E, F, or H) and selected for Ad12-specific sequences by hybridization to Ad12 DNA programmed the synthesis of virion proteins II (hexon), III (penton base), IV (fiber), and V (minor core protein). The other polypeptides presumably related to virion polypeptides VI, VII, and VIII did not comigrate with the viral marker proteins which were prepared from purified Ad12 particles (Fig. 3, lanes A and G). It has been shown in the Ad2 system that in vivo virion polypeptides VI, VII, and VIII are derived from precursor polypeptides of higher molecular weights (1, 27). Similar precursors might also exist in Ad12-infected cells, and we, therefore, considered it likely that unmatched polypeptides of higher molecular weights represented precursors to polypeptides

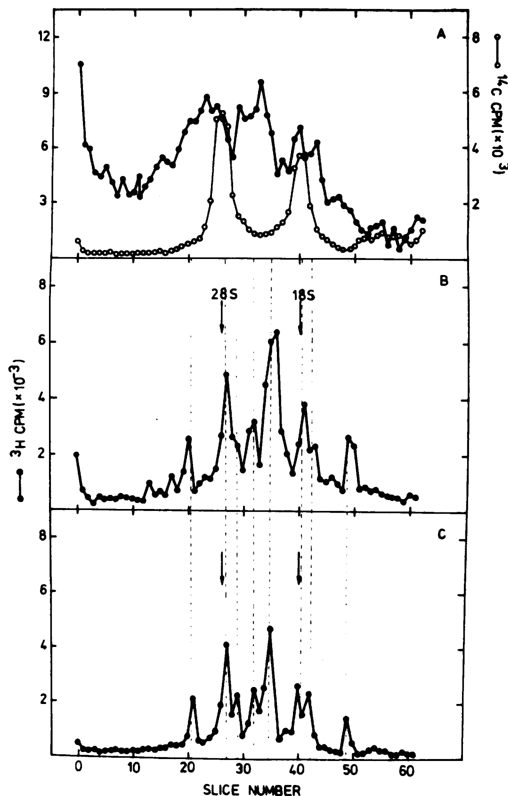

FIG. 2. *Analysis by polyacrylamide gel electrophoresis of cytoplasmic RNA prepared from KB cells after infection with Ad12. RNA was labeled at 30 to 32 h postinfection and prepared as described in the text. The RNA was subjected to electrophoresis in 3.5% polyacrylamide gels in 98% formamide-0.02 M sodium phosphate at room temperature and 100 V for 16 h. At the end of electrophoresis, the cylindrical gels were cut into slices, and each gel slice was eluted with 1 ml of 50% formamide-0.1% SDS-0.75 M NaCl-0.075 M sodium citrate for 24 h at 37°C. Equal portions of each sample were counted in a liquid scintillation spectrometer. (A) Unselected RNA; (B) RNA selected by one step of hybridization (see Table 1); and (C) RNA selected by two steps of hybridization against Ad12 DNA. Symbols: ●, ^{3}H-labeled cytoplasmic RNA; ○, ^{14}C-labeled rRNA used as molecular weight marker. The positions of the 28S and 18S rRNA are indicated by vertical arrows.*

VI, VII, and VIII. This interpretation was supported by the finding that the proteins with molecular weights of 34,000 (34K), 27K, and 21K could be immunoprecipitated with antisera prepared against Ad12 virions (Fig. 3, lane J). Protein IX, a hexon-associated component, was synthesized in such low amounts that it was not detected in the autoradiograms. However, this protein could be labeled with [^{14}C]leucine and was detected when gels were cut into individual slices and radioactivity was measured in each slice (data not shown). In addition to the structural proteins, polypeptides of molecular weights 90K, 55K, 38K, 14K, 11.5K, and 9K were also produced in the in vitro translation system (Fig. 3, lanes E, F, and H). These bands were observed only when the RNA used as message was selected by hybridization to Ad12 DNA.

It is conceivable that the polypeptides with molecular weights 38K, 14K, 11.5K, and 9K constitute products of premature termination of cell-free translation.

The molecular weights of the late Ad12 proteins were estimated by comparison with the well-characterized polypeptides of Ad2 (Fig. 1) and with a set of marker proteins with known molecular weights. When the Ad12-specific RNA isolated at 30 or 40 h postinfection from Ad12-infected KB cells was translated in an S-30 extract of wheat germ, the same polypeptides were produced (Fig. 4, lanes C and D).

Cell-free synthesis programmed by early Ad12-specific RNA isolated from Ad12-infected permissive KB cells. Early viral RNA used to program cell-free synthesis was isolated at 10 h postinfection of KB cells with Ad12. As described above, the total cytoplasmic RNA was extracted and preselected for Ad12-specific RNA by hybridization to Ad12 DNA. To increase the amount of early viral RNA synthesized in the cells, 20 μg of cycloheximide per ml was added at 2 h postinfection to prevent the switch from the early to the late phase during permissive infection. In these experiments the RNA was prepared at 16 to 18 h postinfection.

The analysis of the products of the reticulocyte cell-free translation system programmed by early viral RNA revealed distinct polypeptides with molecular weights of 68K, 50K, 42K, 39K, 34K, 21K, 19K, 13K, 12K, and 10K (Fig. 5, lane C). Improved results were obtained when the RNA was preselected twice on Ad12 DNA (Fig. 5, lane D). When RNA from mock-infected cells was preselected on Ad12 DNA (Fig. 5, lane E) or when RNA from Ad12-infected KB cells was preselected on lambda DNA (data not shown), these polypeptides were not synthesized. At least five of these early polypeptides could be precipitated by immune sera prepared from Ad12 tumor-bearing hamsters.

Translation of viral RNA prepared from abortively infected BHK-21 cells. RNA for in vitro translation was isolated from Ad12-infected BHK-21 cells at 12 h postinfection. The polypeptides synthesized with this RNA had molecular weights of 68K, 61K, 50K, 42K, 39K, 37K, 21K, 14K, 13K, 12K, 11K, and 10K (Fig. 6, lane D). The significance of the 105K poly-

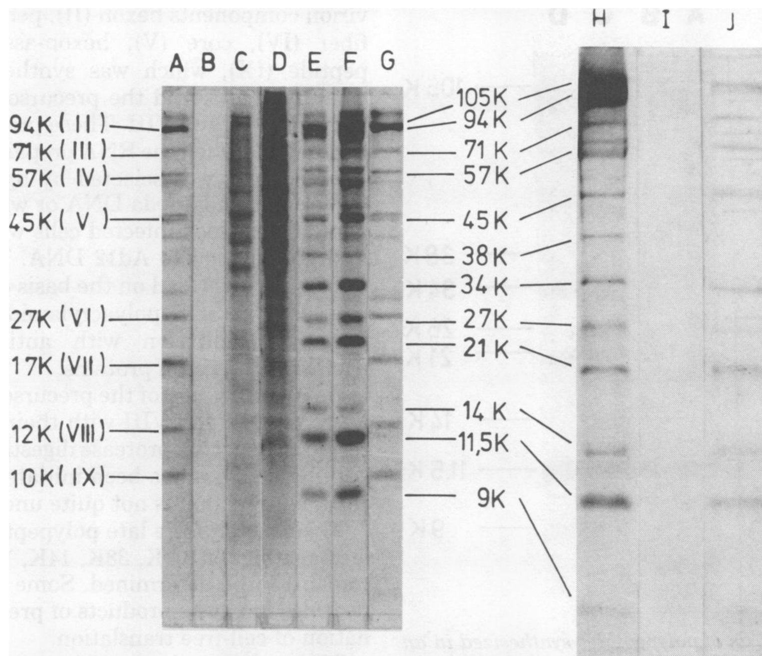

FIG. 3. *Analysis of polypeptides synthesized in the reticulocyte in vitro translation system programmed with Ad12 late mRNA. The gel compares [^{35}S]methionine-labeled purified Ad12 proteins (lanes A and G) with reaction products of a cell-free protein-synthesizing system. (B) No RNA added. The reticulocyte cell-free system was programmed with (C) 15 μg of unselected cytoplasmic RNA from Ad12-infected KB cells, (E) 15 μg of preselected Ad12 RNA prepared from Ad12-infected KB cells at 30 h postinfection, (F) 38 h postinfection, and (H) 46 h postinfection. (D) Viral proteins labeled for 2 h in vivo at 38 h postinfection. (I) Immunoprecipitated proteins from a cell-free system to which no RNA was added and (J) from a cell-free system programmed with preselected RNA from infected KB cells prepared at 38 h postinfection. For immunoprecipitation, a rabbit antiserum directed against Ad12 particle proteins was used. RNA was selected by hybridization to Ad12 DNA and was translated in cell-free extracts. Translation products were analyzed by polyacrylamide gel electrophoresis on 13% SDS—polyacrylamide slab gels. Autoradiograms were exposed for 3 days.*

peptide, which was also found in isolated purified virions (Fig. 3, lanes A and G), is unknown.

Most of these polypeptides were very similar to those synthesized under the direction of RNA isolated from infected permissive KB cells (Fig. 4), except the 34K protein which could not be detected when RNA isolated from Ad12-infected BHK-21 cells was used. In most experiments we observed additional polypeptides of 37K, 14K, and 11K molecular weights (Fig. 6, lane D), which were occasionally seen as minor bands in gels in which the in vitro products of translation experiments were analyzed after programming with RNA prepared from infected KB cells. At least six early polypeptides coded by viral RNA prepared from infected BHK-21 cells could be immunoprecipitated (Fig. 6, lane E) by antisera from Ad12 tumor-bearing hamsters.

Cell-free translation of viral RNA isolated from the Ad12-transformed hamster cell line HA12/7. Ad12-specific RNA from HA12/7 cells growing in suspension cultures was prepared as described above. Before viral RNA was added to the cell-free system, Ad12-specific RNA was selected twice by hybridization to Ad12 DNA. Analysis of the products of cell-free protein synthesis revealed that distinct polypeptides of molecular weights 68K, 50K, 48K, 42K, 37K, 21K, 13K, 10K, and 9K (Fig. 7, lanes D, F, and H) were synthesized. Lanes E, G, and I show the polypeptides which could be immunoprecipitated by three different antisera prepared from hamsters bearing tumors induced by subcutaneous injections of HA12/7 cells. The peptide patterns observed resembled those which were obtained when early viral RNA prepared from Ad12-infected KB or BHK-21 cells was translated in an in vitro system. The 39K, 34K, and 12K polypeptides which were found by using early viral RNA from infected cells could not be detected when RNA from HA12/7 cells was translated. RNA from HA12/7 cells, however,

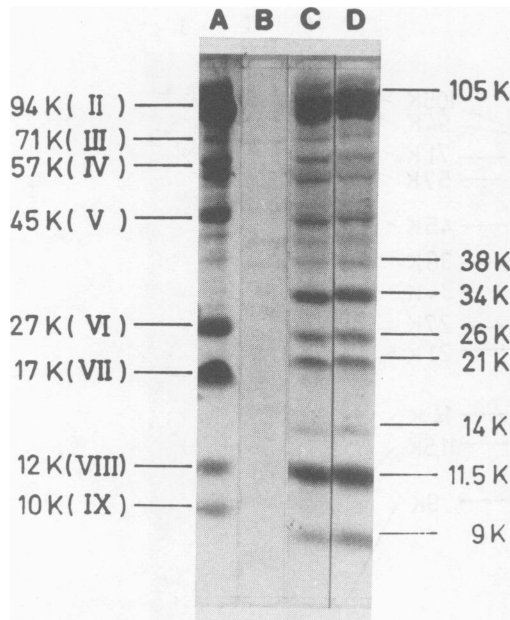

FIG. 4. *Analysis of polypeptides synthesized in an S-30 extract prepared from wheat germ and programmed with Ad12 late mRNA. (A) [^{35}S]methionine-labeled Ad12 virion proteins; (B) polypeptides synthesized in vitro, when no RNA was added; (C) polypeptides synthesized after the addition of 15 μg of preselected Ad12 RNA prepared from Ad12-infected KB cells at 30 h or (D) 40 h postinfection. Procedures for hybridization, cell-free synthesis, and gel electrophoresis were described in the text. Polypeptides were subjected to electrophoresis in 13% SDS-polyacrylamide slab gels; the autoradiogram was exposed for 3 days.*

gave rise to the synthesis of a 48K protein which was not produced when RNA from infected cells was used.

DISCUSSION

In this report, we have described the in vitro translation of Ad12-specific RNA prepared from Ad12-infected human cells and from one Ad12-transformed hamster cell line. The RNA used to program cell-free protein synthesis was prepared from the cytoplasm and was selected as virus-specific RNA by hybridization to Ad12 DNA. In some experiments, viral RNA was selected for polyadenylic acid sequences by chromatography on polyuridylic acid-Sepharose before hybridization to Ad12 DNA. The results of in vitro translation experiments were practically identical regardless of whether the RNA used was selected on polyuridylic acid-Sepharose.

The Ad12-specific RNA obtained late after infection stimulated cell-free synthesis of at least 12 polypeptides. Among these were the principal virion components hexon (II), penton base (III), fiber (IV), core (V), hexon-associated polypeptide (IX), which was synthesized in very small amounts, and the precursors of polypeptides VI, VII, and VIII. These proteins were not synthesized when the RNA prepared from Ad12-infected cells was selected by hybridization to bacteriophage lambda DNA or when RNA prepared from mock-infected cells was preselected by hybridization to Ad12 DNA. The viral proteins were identified on the basis of their rate of migration in SDS-polyacrylamide gels and by immunoprecipitation with antisera directed against Ad12 virion proteins.

An identification of the precursors of polypeptides VI, VII, and VIII with their in vivo counterparts by partial protease digestion and tryptic peptide analysis has been initiated. At present, this identification is not quite unequivocal.

The nature of the late polypeptides with molecular weights 105K, 38K, 14K, 11.5K, and 9K remains to be determined. Some of these polypeptides might be products of premature termination of cell-free translation.

There seem to be no stringent limitations as to the source of the translational components, since the wheat germ and the reticulocyte systems gave qualitatively similar results.

Cell-free translation of Ad12-specific RNA obtained from productively infected KB cells early after infection yielded 10 polypeptides with molecular weights of 68K, 50K, 42K, 39K, 34K, 21K, 19K, 13K, 12K, and 10K. The early viral RNA used to program cell-free synthesis was selected twice by hybridization to Ad12 DNA. Thus, the RNA coding for these polypeptides was Ad12 specific, and the polypeptides translated in vitro were presumably virus and not host specified. Furthermore, most of the early polypeptides synthesized in vitro could be immunoprecipitated by antisera prepared from Ad12 tumor-bearing hamsters.

In all experiments reported here, [^{35}S]methionine was used as label, and hence we would have missed any polypeptides not containing methionine. One experiment using [^{3}H]leucine instead of [^{35}S]methionine did not reveal any additional early proteins.

Polypeptides of almost identical molecular weights were synthesized in vitro by using virus-specific RNA prepared early after the abortive infection of BHK-21 cells with Ad12. The 34K protein could not, however, be detected in several in vitro translation experiments using different RNA preparations from Ad12-infected BHK-21 cells. Could this 34K protein play a role in the abortiveness of the BHK-21-Ad12 system? In comparison with proteins produced in

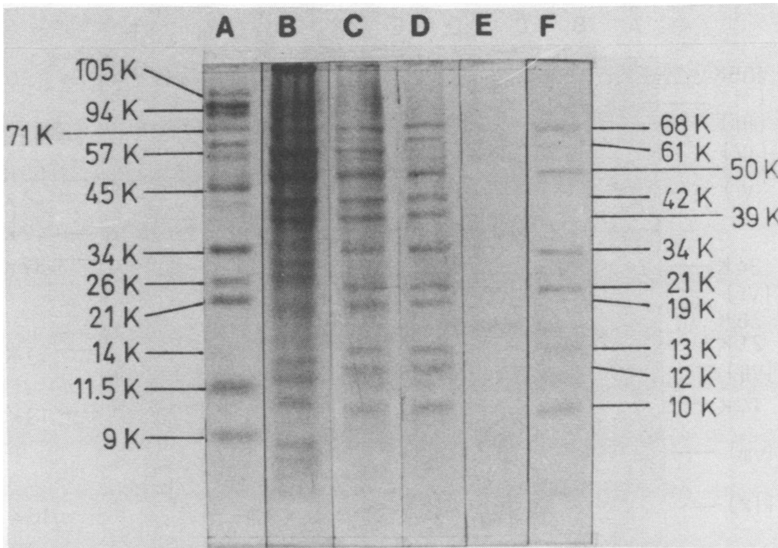

FIG. 5. *Analysis of the products of cell-free protein synthesis programmed by early Ad12-specific RNA isolated from infected KB cells at 10 h postinfection. (A) Polypeptides synthesized in the presence of preselected late Ad12 RNA prepared at 40 h postinfection, (B) unselected early RNA, (C) early RNA selected once, and (D) early RNA selected twice by hybridization to Ad12 DNA; (E) RNA from mock-infected cells preselected once on Ad12 DNA; (F) immunoprecipitated polypeptides from a cell-free translation system programmed with early viral RNA preselected twice. The immune serum used was prepared from Ad12 tumor-bearing hamsters. A total of 10 to 15 μg of RNA per 100 μl of reaction mixture was used to program cell-free protein synthesis. Procedures for hybridization, cell-free protein synthesis, and gel electrophoresis were described in the text. The autoradiogram was exposed for 5 days.*

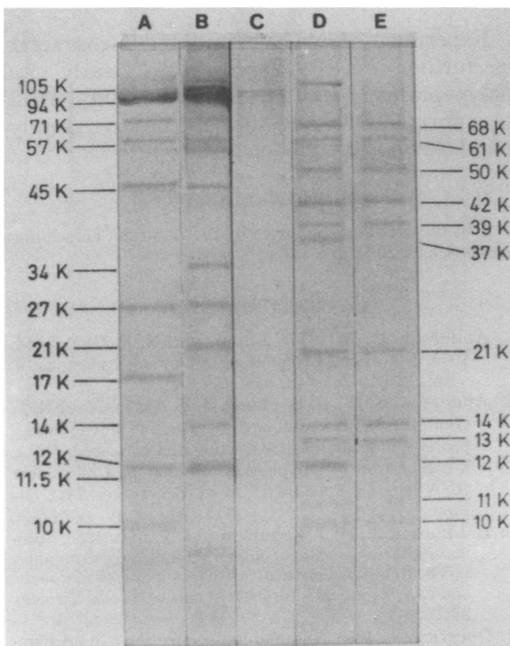

FIG. 6. *Analysis of the products of cell-free protein synthesis programmed by Ad12-specific RNA isolated from infected BHK-21 cells early postinfection. (A) In vivo [^{35}S]methionine-labeled Ad12 virion proteins. The reticulocyte in vitro translation system was programmed with (B) 15 μg of preselected late Ad12 RNA prepared from infected KB cells, (C) no added RNA, (D) 10 μg of viral RNA prepared from Ad12-infected BHK-21 cells at 12 h postinfection and preselected once by hybridization to Ad12 DNA; (E) immunoprecipitated polypeptides from the cell-free system programmed with preselected early Ad12 RNA isolated from infected BHK-21 cells. The immune serum used was prepared from an Ad12 tumor-bearing hamster. Procedures for hybridization, cell-free protein synthesis, and gel electrophoresis were described in the text. The autoradiogram was exposed for 6 days.*

cell-free systems directed by early viral RNA from productively infected cells, additional proteins of 61K, 37K, 14K, and 11K molecular weights were observed with preselected viral RNA isolated from Ad12-infected BHK-21 cells. The early 61K protein had an electrophoretic mobility very similar to the Ad12-coded DNA-binding protein discovered in vivo by Rosenwirth et al. (32).

A comparison of the polypeptides synthesized in the reticulocyte cell-free system under the direction of early RNA from Ad12-infected cells with those produced under the influence of preselected viral RNA isolated from the Ad12-transformed cell line HA12/7 revealed polypep-

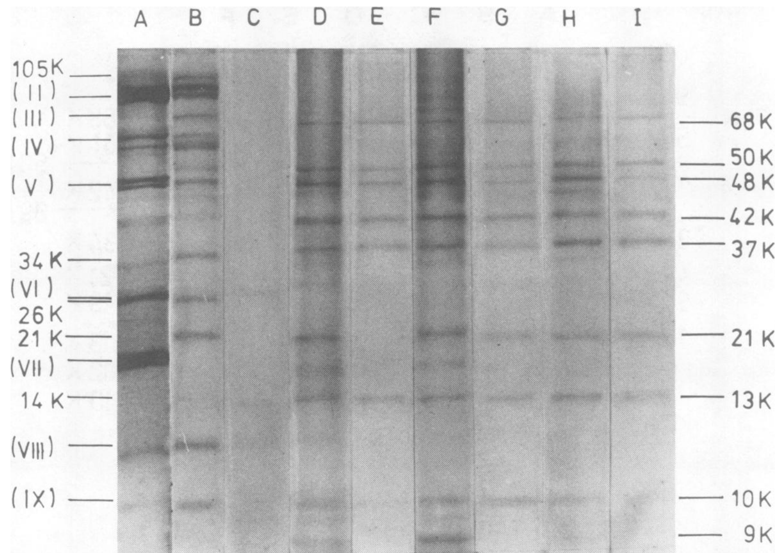

FIG. 7. *Analysis of the products of the reticulocyte cell-free translation system programmed with Ad12 RNA isolated from Ad12-transformed HA12/7 cells. (A) Ad12 virion proteins; (B) polypeptides synthesized after the addition of 15 μg of preselected Ad12 late RNA prepared from infected KB cells; (C) no RNA added; (D, F, and H) polypeptides synthesized in three independent experiments in which Ad12-specific RNA from HA12/7 cells was translated in vitro; (E, G, and I) polypeptide patterns after immunoprecipitation of the proteins synthesized in extracts characterized in (D), (F), and (H), respectively. Antisera prepared from three different Ad12 tumor-bearing hamsters were used. Procedures for hybridization, cell-free protein synthesis, and gel electrophoresis were described in the text. The polypeptides were subjected to electrophoresis on 13% SDS-acrylamide slab gels; the autoradiogram was exposed for 5 days.*

tides almost identical in size, except for two proteins: (i) a new polypeptide of 48K molecular weight could be observed; (ii) three polypeptides coded by early viral RNA prepared from infected cells were missing, including the 34K protein which was not synthesized when RNA isolated from abortively infected cells was used. This finding may correspond with the observation by Ortin et al. (26) that the early region E3 of the Ad12 genome is not expressed in HA12/7 cells.

The differences in protein patterns obtained with early Ad12 RNA prepared from infected KB cells and from Ad12-infected BHK-21 cells or HA12/7 cells were probably not due to the fact that cycloheximide enhancement was used in Ad12-infected KB cells. Experiments without the use of cycloheximide yielded essentially the same results.

The RNA prepared from the HA12/7 cells also contained information for a few minor polypeptides which we were not able to detect in in vitro translation experiments with viral RNA isolated either early or late from productively infected KB cells. Whether these minor species were distinct polypeptides or artifacts of the cell-free translation system could not be determined at this time.

Experiments have been initiated to characterize further the in vitro-synthesized early viral polypeptides from Ad12-infected and Ad12-transformed cells and to map these viral polypeptides on the viral genome.

ACKNOWLEDGMENT

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