Journal of Virology, Aug. 1972, p. 220–227
Vol. 10, No. 2
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Synthesis of Viral Ribonucleic Acid During Restricted Adenovirus Infection

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Received for publication 29 March 1972

The mechanism by which simian virus 40 converts the abortive adenovirus type 7 infection of monkey cells into an efficient lytic infection has been investigated. Analysis of ribonucleic acid (RNA) synthesis during unenhanced and enhanced infection of monkey cells has shown that adenovirus RNA synthesized in the abortive infection contains both "early" and "late" sequences. In hybridization competition experiments, early adenovirus RNA from human cells prevented the hybridization of only 20% of the adenovirus RNA transcribed in unenhanced infection. Further, the RNA from unenhanced cells was able to completely block the hybridization of RNA synthesized during enhanced infection. Finally, virus-associated RNA, which is a late RNA transcribed in lytic adenovirus infection, is also produced in the unenhanced infection. An accompanying paper describes a marked deficiency in adenoviral capsid protein synthesis in the unenhanced infection. We conclude that RNA sequences, which are sufficient to code for the synthesis and assembly of structural proteins of adenovirus, are transcribed but are not efficiently translated in the unenhanced adenovirus infection of monkey cells.

Several human adenoviruses have been shown to abortively infect monkey kidney cells (22). The virus particles penetrate these cells and are uncoated, and at least some early proteins are synthesized (6, 7, 23–25). Although the viral genome is replicated, very few progeny virus particles are produced (4, 24).

In the presence of simian virus 40 (SV40), the abortive infection of African green monkey kidney (AGMK) cells by human adenovirus is converted to an efficient lytic, replicative cycle (17, 22). The ability of SV40 genetic material to increase the yield of adenovirus by 100- to 1,000-fold has been termed "enhancement" (22, 25). An earlier study demonstrated that two- to three-fold more ribonucleic acid (RNA) hybridizable to adenovirus deoxyribonucleic acid is found in the enhanced than in the unenhanced infection (4).

In an accompanying report (3), it was demonstrated that little adenovirus structural protein could be detected after unenhanced (adenovirus only) infection of monkey cells. Since some late functions appear to be carried out inefficiently, we have investigated in the present study the types of virus-specific RNA synthesized after adenovirus type 7 (Ad7) infection of monkey kidney cells. This has been done through comparison of the messenger RNA (mRNA) of enhanced and unenhanced infection by hybridization competition and through attempts to detect the presence of a low-molecular-weight (6*S*) RNA, associated with the late stages of adenovirus replication, termed virus-associated RNA (VA RNA) (19, 20, 26).

MATERIALS AND METHODS

Cells. Primary AGMK and primary human embryonic kidney (HEK) cells were obtained from Flow Laboratories (Rockville, Md.). CV-1 cells, an epithelial subclone of AGMK cells isolated by J. Robb, were obtained from J. Maio. The CV-1 and primary AGMK cell lines were chosen for study because coinfection of these cells with SV40 and adenovirus type 2 (Ad2) or Ad7 yields at least 1,000-fold more adenovirus than if these cells are singly infected with adenovirus (3). KB cells, obtained from American Type Culture Collection, were maintained in Eagle medium containing 5% fetal calf serum (GIBCO, Grand Island, N.Y.). Monolayer cultures of CV-1 and AGMK cells were maintained as described elsewhere (3). Cells and virus stocks were assayed for contamination with mycoplasma and adeno-associated virus and were found free of these agents.

Viruses. The E46$^-$ preparation of Ad7 (27, 28) and the strain 777 of SV40 (5) were used at a multiplicity of infection of 50 and 10 plaque-forming units (PFU)/cell, respectively, unless otherwise specified. The titer of adenovirus stocks was determined by plaque assay on HEK monolayers (4), and that of SV40 stocks by

plaque titration on AGMK or CV-1 monolayers. Infection of KB cell suspensions has been previously described (2).

Preparation of RNA. [^{3}H]uridine (specific activity 20 Ci/mmole), or [^{14}C]uridine (specific activity 53.1 mCi/mmole), was obtained from Schwarz BioResearch (Orangeburg, N.Y.). After viral infection, cytoplasmic RNA was isolated by treating cell concentrates with 0.75% Nonidet P-40 followed by centrifugation at 600 × *g* for 3 min to remove nuclei (9). Phenol extraction of the cytoplasmic supernatant fluid at 64 C (29) and ethanol precipitation (2) were employed to purify the RNA.

RNA from adenovirus-infected CV-1 cells (unenhanced and enhanced mRNA). Both radioactively labeled and unlabeled RNA were prepared from unenhanced and enhanced adenovirus infection of monkey cells. [^{3}H]-labeled RNA was extracted from CV-1 cells after infection with either Ad7, or Ad7 and SV40, as described above. In each case, 10^8 cells were infected and, 4 hr later, unadsorbed virus was removed by two changes of medium. Forty hours after infection, medium was again removed and 1 mCi of [^{3}H]uridine was added in 50 ml of medium containing 2% dialyzed agammaglobulinic calf serum. Four hours later, the cells were washed twice with chilled Earle solution and were harvested by scraping. At the time of harvest, 1 mg of yeast RNA (Calbiochem, Pasadena, Calif.) was added as carrier to the cells, followed immediately by the addition of Nonidet P-40 detergent. RNA was prepared as described above.

To prepare unlabeled RNA, 8 × 10^8 CV-1 cells were infected with Ad7 at a multiplicity of 40 PFU/cell as described previously (4). Forty-eight hours later, the cells were washed twice with chilled Earle solution and harvested by scraping. RNA was extracted as described above.

Early mRNA. Early adenovirus mRNA was prepared by a modification of the method of Fujinaga and Green (8). A suspension culture of 2 × 10^9 KB cells was concentrated and infected with Ad7 as described previously (2), except that cytosine arabinoside at a concentration of 20 μg/ml (Sigma, St. Louis, Mo.) was added to the medium to prevent DNA synthesis. The cells were harvested by centrifugation and washed twice with chilled Earle solution 10 hr after infection. RNA was prepared as described above.

VA RNA. To examine one late species of low-molecular-weight adenovirus RNA, 10^8 primary AGMK or CV-1 cells were infected with Ad7, or SV40 and Ad7. At 36 or 44 hr after infection, the cells were labeled with [^{3}H]uridine (10 μCi/ml) for 4 hr, and RNA was prepared. To isolate VA RNA from lytic infection of human cells, 5 × 10^8 KB cells were labeled with [^{14}C]uridine (2 μCi/ml) from 17 to 21 hr after infection. The cells were then harvested by centrifugation and the RNA was prepared.

Ad7 Complementary RNA (cRNA) and uninfected cell RNA. The preparation of Ad7 cRNA from Ad7 DNA in vitro with *Escherichia coli* polymerase and [^{3}H]nucleotide triphosphates has been described (2). The preparation of RNA from uninfected CV-1 and uninfected KB cells was done as described above, except that the cells were mock infected with medium only.

DNA-RNA hybridization. Ad7 DNA was prepared from purified virus and was immobilized on nitrocellulose filters as described previously (2). DNA-RNA hybridization was performed by the method of Soeiro and Darnell (30), except in the case of hybridization of VA RNA. Hybridization competition experiments were done according to a modification of methods described previously (2). After incubation of unlabeled RNA with DNA fixed to filters for 18 hr at 64 C, the unlabeled RNA solution was removed from the vial and saved at 5 C. After the filters had been washed twice at 64 C for 45 min, the unlabeled RNA was returned to the appropriate vials, and the radioactively labeled RNA was then introduced into each vial. Incubation at 64 C was continued for an additional 20 hr and the filters were then processed as described by Soeiro and Darnell (30). In experiments involving hybridization competition by unlabeled unenhanced RNA, filters containing 0.25 μg of Ad7 DNA and an amount of radioactive RNA sufficient to saturate this DNA were used. In experiments involving hybridization competition by unlabeled early RNA, filters containing 10 times more Ad7 DNA (2.5 μg) were employed to accurately detect the hybridization of early RNA. Because of the amount of Ad7 DNA employed, it was not possible to add saturating amounts of late enhanced and unenhanced RNA.

In the case of VA RNA, hybridization of RNA to DNA was performed by a modification of the methods of McConaughy, Laird, and McCarthy (13) by utilizing 40% formamide (Eastman) and 2 × SSC (0.30 M sodium chloride, 0.030 M sodium citrate) for 20 hr at 39 C for 30 min, followed by two washes with 2 × SSC at 64 C and digestion with pancreatic and T1 ribonuclease as described previously (2).

Gel electrophoresis. Acrylamide gels of 10% concentration were prepared containing a final concentration of 2 × 10^{-3} M ethylenediaminetetraacetic acid at *p*H 7.0, as described by Maizel (14). Electrophoresis of radioactively labeled RNA was performed at 80 v for 20 hr. The gels were then fractionated by extrusion through a Savant gel divider. The [^{14}C] and [^{3}H] radioactivity of each fraction was assayed using a Beckman scintillation counter.

RESULTS

Species of virus-specific RNA synthesized in enhanced and unenhanced infections. Hybridization competition techniques were used to compare the species of virus-specific RNA synthesized after the abortive infection of monkey kidney cells by Ad7 alone, with the species of RNA synthesized after coinfection by Ad7 and SV40. Unlabeled early Ad7 mRNA was prepared from cytoplasmic extracts of KB cells infected in the presence of cytosine arabinoside (20 μg/ml). Increasing amounts of this unlabeled early RNA were added to filters containing adenovirus DNA, and the filters were then exposed to labeled RNA isolated from the cytoplasm of singly or doubly infected monkey cells. Early adenovirus RNA blocked the hybridization of only 20% of the unenhanced RNA (Fig. 1). The hybridization

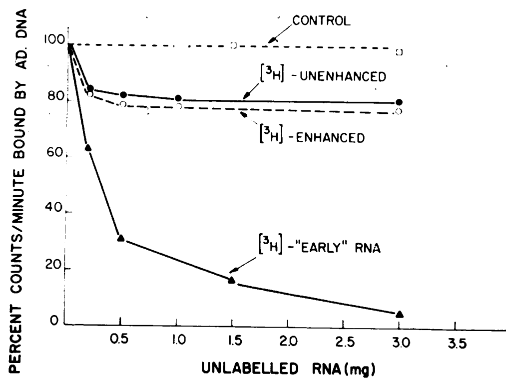

FIG. 1. *Comparison of unenhanced and enhanced RNA: hybridization competition by unlabeled early adenovirus RNA. Filters containing 2.5 μg of Ad7 DNA were first incubated at 64 C for 18 hr with different amounts of unlabeled RNA from KB cells which had been infected with Ad7 in the presence of cytosine arabinoside (early Ad RNA). The filters were washed twice at 64 C, as described in the Materials and Methods section, and one of the following radioactively labeled RNA species was added to the incubation mixture: (i) [^{3}H]-unenhanced RNA obtained from CV-1 cells infected with Ad7 (●); (ii) [^{3}H]-enhanced RNA obtained from CV-1 cells after infected with both Ad7 and SV40 (○); (iii) [^{3}H] early RNA obtained from HEK cells infected with Ad7 in the presence of cytosine arabinoside (▲); (iv) for the control (□), Ad7 DNA filters were first exposed to unlabeled RNA obtained from KB cells mock infected in the presence of cytosine arabinoside. The filters were then processed as described above, and [^{3}H]RNA obtained from CV-1 cells infected with Ad7 was added. The counts per minute of input RNA and counts per minute bound to Ad7 filters in the absence of unlabeled RNA were as follows: (i) input 280,000 counts/min; bound 881 counts/min; (ii) input 620,000 counts/min; bound 1,803 counts/min; (iii) input 805,000 counts/min; bound 2,461 counts/min; (iv) input 280,000 counts/min; bound 881 counts/min.*

of RNA from enhanced infection was also inhibited to the extent of only 20% by competition with unlabeled early adenovirus mRNA. Since coinfection with Ad7 and SV40 yields efficient replication of adenovirus, the RNA obtained from the enhanced infection was assumed to contain all late RNA necessary for synthesis and assembly of virus. In contrast, when DNA synthesis in human cells was blocked with cytosine arabinoside, infection with Ad7 yielded RNA whose hybridization could be inhibited to an extent greater than 90% by early mRNA. No inhibition of hybridization was noted when unlabeled RNA from cytosine arabinoside-treated uninfected KB cells was used.

To directly compare the sequences transcribed after adenovirus infection with or without SV40 coinfection, hybridization competition studies were performed with unlabeled unenhanced RNA (Fig. 2). The unlabeled unenhanced RNA was able to completely inhibit the hybridization of the enhanced RNA to adenovirus DNA. A similar degree of inhibition was noted when unlabeled unenhanced RNA was used in competition with labeled unenhanced RNA (competition against self).

Presence of virus-associated RNA in unenhanced infection. Weissman and Ohe (18–20) have described a low-molecular-weight (6*S*) RNA that is found in the cytoplasm of KB cells late after infection with Ad2 or Ad7. Since hybridization competition experiments indicated that at least some late RNA sequences were present in the unenhanced monkey kidney cell infection, gel electrophoresis was used to look for a specific late adenovirus product, i.e., the VA RNA. Forty hours after infection, Ad7-infected KB cells contain a species of RNA not found in uninfected KB cells or in uninfected AGMK cells (Fig. 3). This species of RNA, migrating at a position corresponding to about 6*S*, is denoted by an arrow. Figure 4 shows the results of an examination of AGMK cells for the presence of VA RNA after infection with SV40

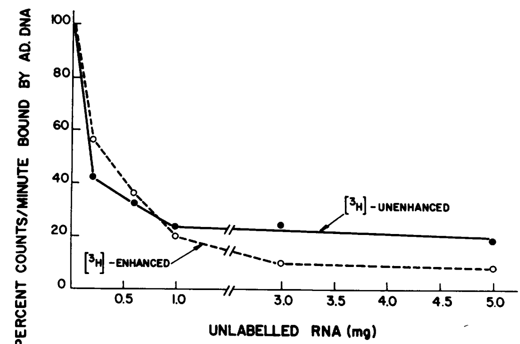

FIG. 2. *Comparison of unenhanced and enhanced RNA: hybridization competition by unlabeled unenhanced adenovirus RNA. Filters containing 0.25 μg of Ad7 DNA were first incubated at 64 C for 16 hr with unlabeled RNA obtained from CV-1 cells infected with Ad7. After washing the filters twice at 64 C, either (i) [^{3}H]-enhanced RNA (○) or (ii) [^{3}H]-unenhanced RNA (●) was added. Counts per minute of input RNA and the counts per minute bound to Ad7 filters in the absence of unlabeled RNA, respectively, were: (i) input 140,000 counts/min; bound 385 counts/min; (ii) input 335,000 counts/min; bound 150 counts/min. The plateaus of counts per minute at maximal competition were comparable to background levels of radioactivity for both of the curves shown in this figure.*

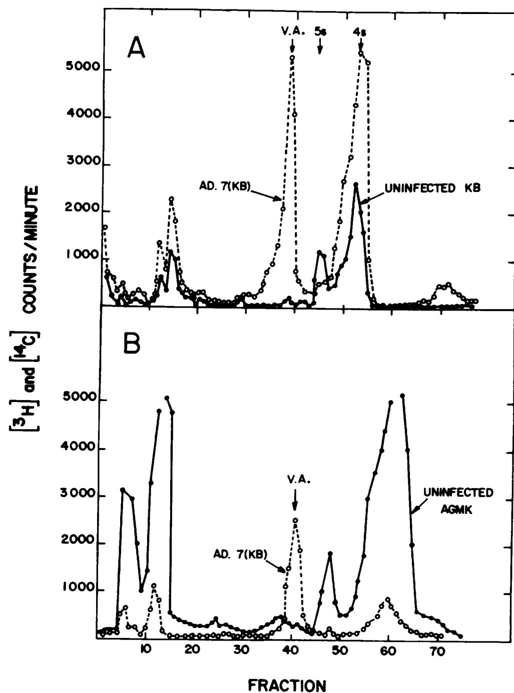

FIG. 3. *Comparison of uninfected monkey and human cells with Ad7-infected human cells with respect to the presence of VA RNA. Electrophoresis on 10% polyacrylamide gels containing 0.1% SDS was carried out at 80 v for 20 hr. In both A and B,* [^{14}C]-*RNA* (○), *obtained from KB cells infected with Ad7 and labeled 17 to 21 hr after infection, was mixed with one of the* [^3H]*RNA species described below immediately prior to electrophoresis. The arrows denote 4S, 5S, and VA RNA migrating at a position corresponding to about 6S. A,* [^3H]*RNA* (●) *from mock-infected AGMK cells, labeled 44 to 48 hr later. B,* [^3H]*RNA* (●) *from mock-infected KB cells, labeled 17 to 21 hr later.*

only, Ad7 only, or both Ad7 and SV40. AGMK cells infected with either Ad7, or coinfected with Ad7 and SV40, contain a 6*S* RNA species which comigrates electrophoretically with the VA RNA from Ad7-infected KB cells. SV40-infected cells do not show this RNA species. In Fig. 3, only a sample of the cytoplasmic RNA was used for electrophoresis. It can be calculated that the yield of VA RNA from infected AGMK cells was threefold higher in the enhanced than in the unenhanced infection.

CV-1 monkey kidney cells were also examined after adenovirus infection for the presence of VA RNA. CV-1 cells infected by Ad7 with or without SV40 coinfection also contain the 6*S* VA RNA (Fig. 5). However, when the monkey or human cells were infected in the presence of cytosine arabinoside, no prominent peak of VA RNA could be demonstrated. Thus, CV-1 cells, as well as primary AGMK cells, contain this 6*S* late RNA species after unenhanced and enhanced adenovirus infection.

The possibility existed that unenhanced infected monkey cells contained an RNA species

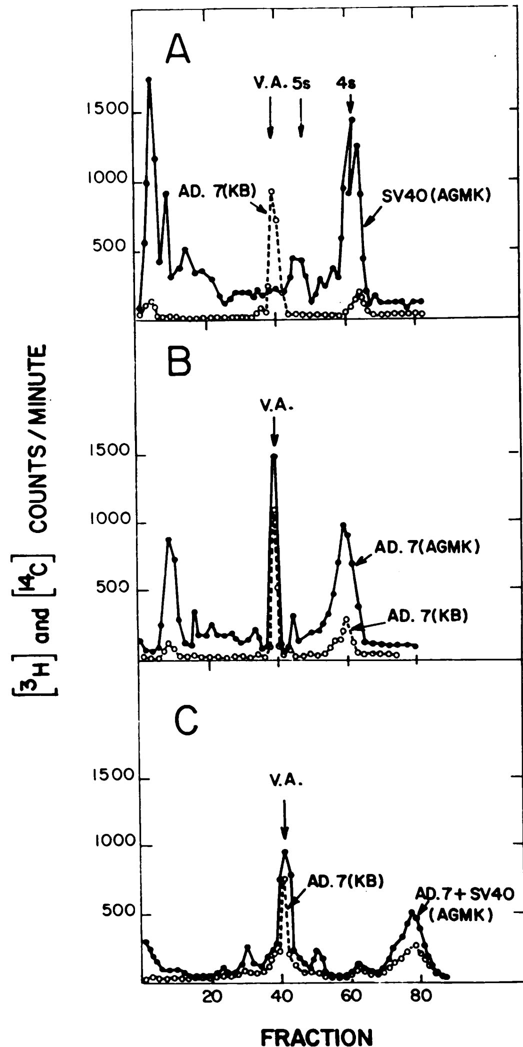

FIG. 4. *Presence of VA RNA in unenhanced and enhanced adenovirus infection of AGMK cells. Electrophoretic conditions and the preparation of* [^{14}C]-*RNA from Ad7-infected KB cells are described in Fig. 3 and the Materials and Methods section.* [^{14}C]*RNA* (○) *and* [^3H]*RNA* (●) *were mixed prior to electrophoresis. A,* [^3H]*RNA* (●) *from AGMK cells infected with SV40 and labeled 44 to 48 hr later. B,* [^3H]*RNA* (●) *from AGMK cells infected with Ad7 only and labeled 44 to 48 hr later. C,* [^3H]*RNA* (●) *from AGMK cells coinfected with Ad7 and SV40 and labeled 44 to 48 hr later.*

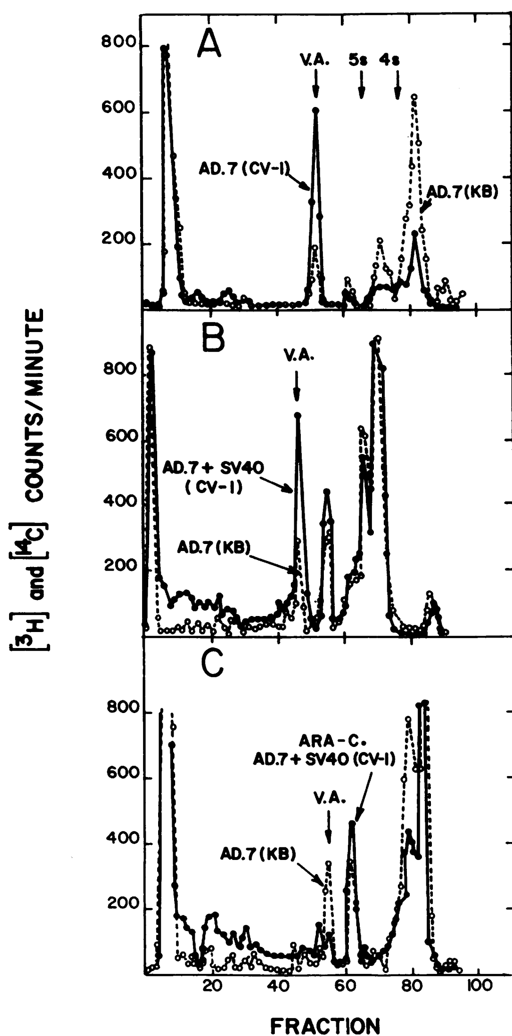

FIG. 5. *Presence of VA RNA in unenhanced and enhanced adenovirus infection of CV-1 cells. Electrophoretic conditions and the preparation of* [^{14}C]-*RNA from Ad7-infected KB cells described in Fig. 3 and the Materials and Methods section.* [^{14}C]*RNA* (○) *and* [^{3}H]*RNA* (●) *were mixed immediately prior to electrophoresis. A,* [^{3}H]*RNA* (●) *from CV-1 cells infected with Ad7 only and labeled 44 to 48 hr later. B,* [^{3}H]*RNA* (●) *from CV-1 cells infected with Ad7 and SV40 and labeled 44 to 48 hr later. C,* [^{3}H]*RNA* (●) *from CV-1 cells infected with Ad7 and SV40 in the presence of cytosine arabinoside (20 μg/ml) and labeled 44 to 48 hours later.*

which fortuitously comigrated with VA RNA from infected KB cells. Therefore, the hybridization properties of the unenhanced 6*S* RNA were studied. Polyacrylamide gel electrophoresis was used to prepare VA RNA from adenovirus-infected KB cells and 6*S* RNA from infected monkey cells. The VA RNA from infected human cells had hybridization properties similar to those of 6*S* RNA obtained from infected monkey cells (Table 1). These RNA species hybridized preferentially to Ad7 DNA, although there was some reaction with host cell (CV-1) DNA. Of greatest significance is the fact that the VA RNA and the 6*S* RNA both showed similar properties of hybridization to Ad7 DNA, as well as to host cell DNA. Although the number of counts per minute hybridized is small, the differences between infected and noninfected cell RNA were statistically significant. These results suggest that a similar species of VA RNA is induced in both adenovirus-infected human and monkey cells, even in the absence of SV40 coinfection.

DISCUSSION

When Ad7 infects monkey kidney cells, an abortive infection ensues in which adenovirus T antigen and adenovirus DNA are synthesized. Since little or no adenoviral structural protein is detected, attempts were made to determine whether or not adenovirus late mRNA is produced, and, therefore, to establish whether the block in replication is at a transcriptional or post-transcriptional level.

An earlier study demonstrated that two- to threefold more radioactive RNA was bound to Ad7 DNA after enhanced infection than after unenhanced infection (4). As indicated in the legends of Fig. 1 and Fig. 2, comparable results were again obtained in this study. This report attempts to expand the previous observations by demonstrating that some late adenovirus RNA sequences are present in the unenhanced infection, although little or no late viral proteins are detectable (2, 4).

The original hypothesis of these experiments was that only early adenoviral mRNA would be present in the unenhanced infection, consistent with the absence of late proteins and with a block in transcription of late mRNA sequences. However, early RNA obtained from cytosine arabinoside-treated adenovirus-infected KB cells was able to inhibit only 20% of the unenhanced RNA species' ability to hybridize to adenovirus DNA. The RNA obtained from late, lytic infection of KB cells by Ad7 was likewise only inhibited 20% by early RNA in hybridization competition experiments (*unpublished data*). These data indicated that perhaps unenhanced RNA contained some late sequences. Another possibility, however, was that the unenhanced RNA contained a different class of early mRNA from that found in cytosine arabinoside-treated

TABLE 1. *Hybridization of virus-associated ribonucleic acid (VA RNA)[a] from infected human and monkey cells*

Type of RNA	Type of DNA on filter	Amount of DNA (μg)	RNA input (counts/min)	RNA bound to filter[b] (counts/min)	Amt bound (%)
VA Ad7 (KB)[c]	Ad7	20	795	68.3 ± 7.6	8.6
VA Ad7 (KB)	CV-1	20	795	38.6 ± 8.0	4.8
6*S*[d] Ad7 (CV-1)	Ad7	20	650	69.1 ± 3.2	10.6
6*S* Ad7 (CV-1)[d]	CV-1	20	650	25.2 ± 2.1	3.84
Uninfected KB	Ad7	10	170,000	11.3 ± 2.3	0.006
Uninfected CV-1	Ad7	10	138,000	10.0 ± 2.3	0.007
Ad7 cRNA[e]	Ad7	10	1,200	402 ± 35	33.4
Ad7 cRNA	CV-1	10	2,000	6 ± 4.1	0.30

[a] A low-molecular-weight RNA (6*S*) found after adenovirus infection.
[b] All hybridizations were done in quadruplicate.
[c] Refers to VA RNA that was prepared from Ad7-infected KB cells. RNA extracts were subjected to electrophoresis on polyacrylamide gels as described in the Materials and Methods section. The gels were fractionated with a Savant gel extruder, and samples were counted to determine the presence of the VA peak.
[d] 6*S* Refers to RNA that was prepared from Ad7-infected CV-1 cells, from polyacrylamide gels as described above.
[e] Complementary RNA.

infected human cells. The existence of different classes of early adenovirus mRNA has been suggested by Lucas and Ginsberg (11). To exclude this latter possibility, unlabeled unenhanced RNA, obtained from adenovirus infected monkey kidney cells, was used to inhibit the hybridization of enhanced and unenhanced RNA to adenovirus DNA. These experiments indicated that RNA species from enhanced and unenhanced infections were quite similar and, thus, that the disparity in protein synthesis previously noted most likely represented a post-transcriptional block in replication. Despite this apparent similarity, the relative insensitivity of hybridization competition techniques (12, 30) implies that the data from these studies cannot be used to exclude the possibility that certain sequences present in the enhanced RNA are not present in the unenhanced RNA. Nonspecific competition could, in part, be due to the fact that unenhanced RNA contains sequences which are related to, but not identical to, those of enhanced RNA. Nevertheless, these similar sequences may serve to inhibit hybridization. With this slight reservation, these data are indicative of considerable sequence similarity between the enhanced RNA and the unenhanced RNA.

A specific late species of adenovirus RNA, the VA RNA, was identified in the unenhanced infection by electrophoresis and hybridization. Previous studies by Ohe and Weissman (20, 26) showed that VA RNA obtained from Ad2- or Ad7-infected KB cells hybridized preferentially to the homologous viral DNA and to a lesser extent to cell DNA. The nucleotide sequence of VA RNA induced after Ad2 infection was shown to be significantly different from that after Ad7 infection. But both showed some sequence similarity to host cell transfer RNA and nucleotide sequences that would permit considerable intramolecular base pairing (20). These observations may partially explain the hybridization of VA RNA to uninfected cell DNA and the low efficiency of hybridization to viral DNA. The greater hybridization to homologous viral DNA (18) and the nucleotide sequence differences (19, 20) suggest that the VA RNA is transcribed from the viral template. This and other possibilities are described by Weissman et al. (19, 20).

Synthesis of structural proteins may utilize up to 30% of the coding capacity of the adenovirus genome (14). A block at the level of transcription likewise would be represented by a 30% decrease in late RNA sequences. Although the hybridization techniques are relatively insensitive, the absence of such a large amount of late RNA would probably have been detected. Ultimately, a firm answer to the question of the identity of RNA species depends on a direct comparison of their nucleotide sequences, or on an in vitro protein-synthesizing system capable of translating the enhanced and unenhanced RNA, followed by analysis of the resulting viral proteins.

In this study, we have primarily investigated the RNA sequences synthesized after infection of monkey cells. Although RNA species from enhanced and unenhanced infection have similar sequences, the possibility exists that these RNA

species may differ from adenovirus RNA transcribed in the course of lytic infection of human cells. This difference, if present, would not reside in the areas of the message coding for structural proteins, since these proteins can be made efficiently in monkey cells coinfected with SV40.

There are a number of reports of post-transcriptional blocks to virus replication. Malamy and co-workers have reported a block at a translational level to replication of bacteriophage T7 (16) or of the bacteriophage ϕII in *E. coli* containing F factor (10). Summers and Jakes (31) have further shown that this RNA is present in translatable form, since the RNA purified from T7-infected *E. coli* which contains the F factor can direct the in vitro synthesis of the late T7 protein lysozyme. Woodson and Joklik (33) have shown that, in HeLa cells treated with isatin-β-thiosemicarbazone, vaccinia viral DNA and both early and late viral RNA were synthesized. However, the late vaccinia RNA sequences were not translated. Ad2 infection of KB cells at an elevated temperature also appears to be restricted at a post-transcriptional level (21, 32).

ACKNOWLEDGMENTS

R. I. Fox is a Medical Scientist Trainee supported by Public Health Service training grant 5T5 GM1674 from the National Institute of General Medical Sciences. S. G. Baum is a Career Scientist of the Health Research Council of the City of New York (I-577).

This project was supported by Public Health Service grants CA 10945 from the National Cancer Institute and AI 405 from the National Institute of Allergy and Infectious Diseases.

We acknowledge helpful discussion with J. Maizel, Jr., M. Horwitz, and S. Weissman.

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