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Transcription and Transport of Virus-Specific Ribonucleic Acids in African Green Monkey Kidney Cells Abortively Infected with Type 2 Adenovirus

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The techniques of deoxyribonucleic acid-ribonucleic acid (DNA-RNA) hybridization and immunological precipitation were used to compare the synthesis of adenovirus-specific macromolecules in African green monkey kidney (AGMK) cells infected with adenovirus, an abortive infection, and coinfected with both adenovirus and simian virus 40 (SV40), which renders the cells permissive for adenovirus replication. When viral protein synthesis was proceeding at its maximum rate, the incorporation of ^{14}C-amino acids into adenovirus structural proteins was about 90 times greater in the doubly infected cells than in cells infected only with adenovirus. However, the rates of synthesis of virus-specific ribonucleic acid appeared to be comparable in the two infections at all times measured. A time-dependent increase in the rate of RNA synthesis observed late in the abortive infection was dependent upon the prior replication of viral DNA. Moreover, all virus-specific RNA species that are normally made late in a productive adenovirus infection (i.e., the true late and class II early RNA species) were also detected in the abortive infection. Adenovirus-specific RNA was detected by molecular hybridization in both the cytoplasm and nuclei of abortively infected cells. Comparable amounts of viral RNA were found in the cytoplasmic fractions of AGMK cells infected either with adenovirus or with both adenovirus and SV40. The results of hybridization-inhibition experiments clearly showed that there was a class of virus-specific RNA molecules, representing about 30% of the total, in the nucleus that was not transported to the cytoplasm. This class of RNA was also identified in similar amounts in productively infected human KB cells. The difference in the abilities of cytoplasmic and nuclear RNA to inhibit the hybridization of virus-specific RNA from whole cells was shown not to be due to a difference in the molecular size of the RNA species from the two cell fractions or to the specific loss of a cytoplasmic species during RNA extraction procedures.

The replication of type 2 adenovirus in African green monkey kidney (AGMK) cells is normally restricted, but the yield of adenovirus can be enhanced by about three orders of magnitude if the cells are co-infected with simian virus 40 (SV40) (2, 8, 17, 18, 24). Previous studies have shown that early adenovirus-specified proteins are synthesized in the abortively infected cells (8, 10, 16). Thus, the virus can adsorb to and penetrate the cells, and its deoxyribonucleic acid (DNA) can be at least partially transcribed. It has also been demonstrated directly that virus-specific ribonucleic acid (RNA) is synthesized (1) and that viral DNA replicates (10, 25, 26). However, only limited amounts of viral capsid proteins are made in relatively few cells (8, 10, 16).

In KB cells productively infected with type 2 adenovirus, the late virus-specific RNA contains species that are not present early in infection (15, 30). Therefore, to determine the molecular basis for the inefficient replication of adenovirus in AGMK cells, it was necessary to discover whether or not all late species of virus-specific RNA are likewise synthesized in the abortive infection. Also, since the transcription of late viral RNA occurs in the nucleus of infected cells whereas translation of messenger RNA takes place in the cytoplasm (29, 31), it was necessary to determine whether the viral RNA made in the

abortive infection could be transported across the nuclear membrane.

The results presented here indicate that the transcription and transport of adenovirus-specific RNA in abortively infected cells are normal in all aspects studied. All species of late virus-specific RNA were synthesized. Moreover, a newly identified species of virus-specific RNA confined to the nucleus of productively infected cells was also observed in the abortive infection. In confirmation of previous reports (8, 10, 16), however, only limited amounts of adenovirus capsid proteins were detected in AGMK cells infected only with adenovirus.

MATERIALS AND METHODS

Virus. The prototype strain of type 2 adenovirus, which had been plaque-purified three times, was used. It was assayed on monolayers of KB cells by the plaque assay (14) or fluorescent focus assay (10). The wild-type strain of SV40 was used. Stocks were grown in AGMK cells and assayed on AGMK cell monolayers by a modification of the plaque assay for adenovirus (14), or by a fluorescent focus assay employing rabbit antiserum prepared against purified SV40 (reference 10; Lucas and Ginsberg, *unpublished results*). The fluorescent focus assay for SV40 could be completed within 3 days; the plaque assay required 2 weeks.

Tissue culture. The growth and maintenance of KB cells were described previously (15). Primary African green monkey (*Cercopithecus aethiops*) kidney cells were obtained from Flow Laboratories, Rockville, Md., and were grown to confluence in 0.5% lactalbumin hydrolysate in Hanks balanced salt solution (Flow Laboratories, H-Lac) supplemented with 2% fetal bovine serum (FBS). The cells were transferred twice in 0.5% lactalbumin hydrolysate in Earle balanced salt solution (Flow Laboratories, E-Lac) supplemented with 2% FBS and then used for experimentation. During experiments, the cells were maintained in Eagle basal medium containing 2% FBS. When 5-fluoro-deoxyuridine (kindly supplied by Hoffman LaRoche, Nutley, N.J.) was employed, it was necessary to dialyze the FBS against 0.15 M NaCl.

Extraction and denaturation of viral DNA. DNA was extracted from purified virus (14) as described previously (3) except that the mercaptoethanol step was omitted. After dialysis of the DNA against 0.1 × SSC (1.0 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), it was denatured by raising the *p*H to 12.8 with 1 N NaOH. The *p*H was adjusted to 7.0 with 1 N HCl after 10 min at room temperature.

Cell fractionation. The cell fractionation method is a modification of that of Hogan and Korner (12) which employs the buffer of Warner et al. (33) and the polyethoxylene alkyl phenol detergent, Triton X-100 (Rohm and Hass). KB cells were harvested by centrifugation, washed once with phosphate-buffered saline (PBS; 0.15 M NaCl, 0.01 M phosphate, *p*H 7.2) and resuspended in Triton lysis buffer [10 mM KCl, 1.5 mM magnesium acetate, 10 mM tris(hydroxymethyl)aminoethane, *p*H 7.5 at 4 C, 1.0% (w v) Triton X-100]. Monolayers of AGMK cells were washed once with PBS, and the Triton lysis buffer was added directly to the cell sheet. After lysis, which occurred almost immediately, the flasks were agitated to remove nuclei from the surface of the vessels. With either cell type, nuclei were pelleted by centrifugation at 800 × *g* for 20 min.

Extraction of RNA. RNA was prepared by using the procedure of Scherrer and Darnell (27) as modified by Homma and Graham (13). Where indicated, RNA was prepared from cytoplasmic extracts by the method of Perry et al. (20) by employing sodium dodecyl sulfate (SDS), phenol, and chloroform. Tritium-labeled RNA was prepared as described previously (15).

Alkaline degradation of RNA. Limited hydrolysis of RNA (5) was performed by adjusting the preparation of purified RNA to 1 N NaOH. After 90 sec at 23 to 27 C, the solution was rapidly adjusted to *p*H 7.0 with HCl, cooled in an ice bath, and dialyzed against 2 × SSC. As shown previously (15), these conditions yield a product with a sedimentation coefficient of 4 to 5*S*.

DNA-RNA hybridization. Methods used for direct hybridization and for hybridization inhibition were described previously in detail (11, 15).

Immunological precipitation of viral proteins. The technique for immunological precipitation of viral proteins is similar to that used previously (3, 31). Monolayers of AGMK cells (about 12.5 × 10^6 cells per flask) were washed with PBS and removed from the surface of the culture vessels by employing Triton X-100 lysing buffer. The solution was diluted to 5 ml with PBS and sonically treated for 2 min (four 30-sec intervals separated by 30-sec cooling periods) by using an MSE sonic oscillator operating at peak amplitude. The samples were centrifuged for 15 min at 4,000 × *g*, and SDS (final concentration of 0.05%) was added to the supernatant fluid. To 0.4-ml quantities of the cell extract was added 0.2 ml of various dilutions of either normal rabbit serum or an antiserum from rabbits immunized with purified type 2 adenovirus, and the mixture was incubated for 2 hr at 37 C. The precipitates were collected by centrifugation for 10 min at 4,000 × *g*, washed twice with 0.6 ml of PBS, and then dissolved in 1 N NaOH. A number of concentrations of antisera were used to ensure that determinations were made in antibody excess. All results were corrected for nonspecific precipitation by normal rabbit antiserum. As previously demonstrated (35), unrelated antigen-antibody complexes did not trap, and hence precipitate, adenovirus proteins. Rabbits were immunized as described previously (10).

RESULTS

Rate of synthesis of adenovirus structural proteins. AGMK cells were infected either with adenovirus or with adenovirus and SV40. At various times after infection, the cells were labeled for 1-hr periods with ^{14}C-amino acids. The

incorporation of label into total protein progressively decreased in the abortive and enhanced infections (Table 1). This decrease was less rapid in the doubly infected cells. When the amount of label incorporated into adenovirus-specific proteins was measured by immunological precipitation, it was observed that much more viral protein was synthesized in the coinfected cells. When viral protein synthesis was proceeding at its maximum rate, about 90 times more label was incorporated into adenovirus proteins in the adenovirus-SV40 infection than in the single infection. In this experiment, co-infection with SV40 enhanced the yield of infectious adenovirus about 1,300-fold. The use of immunoprecipitation permitted a more quantitative determination of protein synthesis than did the previously used methods of complement fixation (10) and immunofluorescence (8, 10, 16). However, the observation that viral protein synthesis was markedly less in the abortive infection was confirmed.

Rate of synthesis of virus-specific RNA. To determine the mechanism for the decreased synthesis of viral structural proteins in the abortive infection, the quantity and nature of the virus-specific RNA made in the abortive and enhanced infections were compared. At various times after infection, cells infected either with adenovirus or adenovirus and SV40 were labeled for 1-hr periods with ^{3}H-uridine. To determine the amount of viral RNA synthesized, the total RNA was extracted from each culture and hybridized with adenovirus DNA. Hybridization was performed with each sample with a constant RNA concentration and varying amounts of DNA. Hence, a series of curves showing the saturation of virus-specific RNA with DNA were constructed. The data were replotted as described previously (15), and the percentage of the label incorporated into virus-specific RNA was determined. These values, plotted as a function of time after infection, are presented in Fig. 1. In both the abortive and enhanced infections, the rate of incorporation increased until about 30 hr after infection, at which time 10 to 20% of the ^{3}H-uridine incorpo-

TABLE 1. *Rate of synthesis of total and adenovirus-specific protein in AGMK cells infected with adenovirus or with adenovirus and SV40*

Infecting virus[a]	Labeling time[b] (hr postinfection)	Counts/min ($\times 10^{-5}$) incorporated[c]	Counts/min ($\times 10^{-4}$) in viral protein[d]	% in viral protein
Adenovirus	24–25	1.02	0.05	0.5
Adenovirus-SV40	24–25	2.31	2.33	10.1
Adenovirus	41–42	0.80	0.06	0.8
Adenovirus-SV40	41–42	1.86	6.01	32.3
Adenovirus	51–52	0.37	0.03	0.8
Adenovirus-SV40	51–52	0.78	2.00	25.6
None		3.43	0.04	0.1

[a] Monolayers of AGMK cells were infected either with 2 fluorescent focus-forming units of type 2 adenovirus per cell or with 2 fluorescent focus-forming units of adenovirus and 10 fluorescent focus-forming units of SV40 per cell.

[b] Each cell culture (12.5×10^6 cells) was labeled with 20 μCi of ^{14}C-amino acids in 1 ml of amino-acid-free media supplemented with normal concentrations of tryptophan and glutamine.

[c] Counts per minute incorporated into protein is that material precipitated by hot trichloroacetic acid.

[d] Viral protein was determined by immunological precipitation by using antiserum prepared against purified type 2 adenovirus. See Materials and Methods for description of procedure.

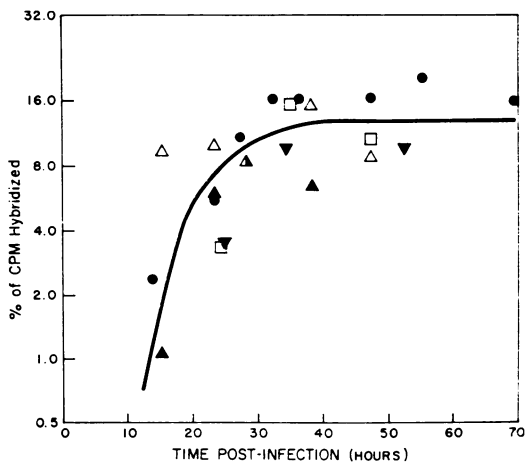

FIG. 1. *Synthesis of adenovirus-specific RNA in AGMK cells infected either with adenovirus (closed symbols) or adenovirus and SV40 (open symbols). Each infected cell culture (12.5×10^6 cells) was labeled with 100 μCi of ^{3}H-uridine for 1-hr periods at various times after infection. RNA was extracted, and a constant amount (from 2 to 8 μg of total RNA) of each sample was hybridized with filter-bound adenovirus DNA ranging in concentration from 2 to 10 μg per filter. ^{3}H-uridine counts, hybridized for the 184 hybridization samples assayed, ranged from 101 to 1,840 counts per min. The average value for nonspecific binding, determined by incubation of the 23 RNA samples with 92 blank filters, was 3 counts per min. The symbols ●, □, ▼, △, ▲ refer to data from four separate experiments. Percentage of counts per minute hybridized is the number of counts per minute hybridized at saturating concentrations of DNA (determined as described in the text), divided by the number of counts per minute added to the hybridization reaction mixture, multiplied by 100.*

rated into RNA was in virus-specific sequences. To demonstrate the degree of variation among experiments, the results of a number of separate experiments are included in Fig. 1. The variation noted was not observed when this assay was applied to an analysis of viral RNA synthesis in productively infected KB cells maintained in suspension culture (15). It may simply reflect a variation in biosynthetic capacity among primary cell monolayer cultures. However, it is important to note that the variations in RNA synthesis were not accompanied by similar variations in production of infectious virus, i.e., the yield of virus was never greater than the input inoculum.

Dependence of virus-specific RNA synthesis on DNA replication. Adenovirus structural proteins are made late in infection, that is, after the initiation of viral DNA replication (9, 34). Likewise, without the initiation of viral DNA synthesis, late virus-specific RNA is not transcribed (3, 15). To determine whether or not the virus-specific RNA made late in the abortive infection of AGMK cells possessed this distinguishing characteristic of late RNA, the following experiment was performed. AGMK cells were infected with adenovirus either in the absence or presence of 2 μM FUdR, an effective inhibitor of DNA synthesis and late RNA transcription (3, 9, 15). The amount of virus-specific RNA synthesized from 20 to 21 hr after infection was determined by DNA-RNA hybridization (Fig. 2). The data were analyzed by using the reciprocal plots described previously (15). In the presence of FUdR, only 0.08% of the ^{3}H-uridine found in RNA labeled at 20 to 21 hr was in virus-specific RNA (Fig. 2D). This presumably represents incorporation into early virus-specific sequences. In the absence of the pyrimidine analogue, the amount was 13.5% (Fig. 2B). The difference between the two numbers suggests that (i) most of the virus-specific RNA normally synthesized at this time in the abortive infection was late RNA, i.e., its synthesis depended upon the prior replication of viral DNA, or (ii) the difference reflected a greatly increased rate of synthesis of early species late in the abortive infection. Since there is a class of early virus-specific RNA (early RNA class II) whose synthesis is greatly enhanced late in the productive adenovirus infection of human KB cells (15), it was necessary to test these possibilities.

Nature of the RNA made late in the abortive infection. RNA prepared from human KB cells harvested late in a productive infection with type 2 adenovirus contains true late virus-specific RNA, which is synthesized only after the initiation of viral DNA replication, and class II

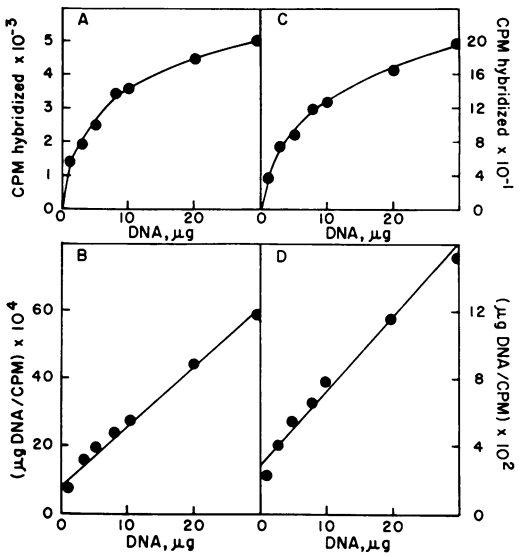

FIG. 2. *Relationship of adenovirus-specific RNA synthesis to viral DNA replication in the abortive infection. AGMK cells were infected with 3 plaque-forming units of type 2 adenovirus per cell, either in the absence (A, B) or presence (C, D) of 2 μM FUdR, and labeled with 500 μCi of ^{3}H-uridine from 20 to 21 hr after infection. RNA was extracted, and each sample was hybridized with varying concentrations of filter-bound adenovirus DNA. B and D are reciprocal plots (see text) of the data presented in A and C, respectively, and were used to calculate the counts per minute hybridized at saturating concentrations of DNA. In A, the input of ^{3}H-RNA for each hybridization reaction mixture was 15.7 μg (4.6 × 10^4 counts per min); in C, the input was 25.8 μg (3.0 × 10^5 counts per min). The reciprocal of the slope of the line shown in B is 6,173 counts per min, whereas that of the line in D is 227 counts per min.*

early RNA, which is made both before and after DNA replication begins (15). To determine whether or not these classes of RNA were made in the abortive infection, hybridization-inhibition experiments were performed. Unlabeled RNA was prepared from abortively infected AGMK cells harvested at 23 hr after infection and from productively infected KB cells harvested at 18 hr after infection. These preparations were used to compare their abilities to inhibit the hybridization of ^{3}H-uridine-labeled RNA extracted from productively infected KB cells harvested at a late time. The two unlabeled RNA preparations inhibited hybridization to the same extent (Fig. 3). Thus, it can be concluded that, within the limits of detection of this assay, all species of true late and class II early RNA were synthesized in the abortive infection of AGMK cells.

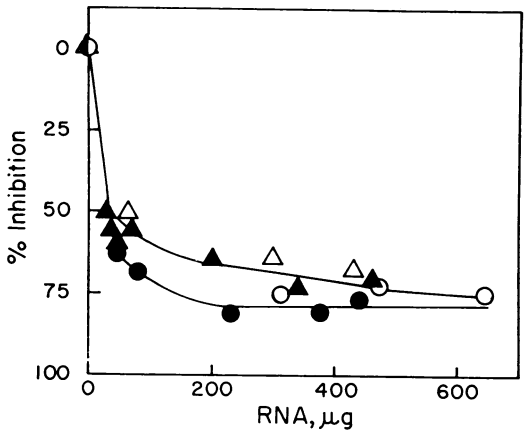

FIG. 3. *Hybridization-inhibition of RNA labeled late in the productive adenovirus infection of KB cells by unlabeled RNA from late in the abortive infection of AGMK cells (▲, △) or from late in the productive infection of KB cells (●, ○). In the experiment designated ● ▲, 0.5 μg of viral DNA per filter was used and late ^{3}H-RNA was prepared from KB cells labeled from 18 to 19 hr after infection with 2 μCi of ^{3}H-uridine per ml. In the experiment designated ○ △, 0.2 μg of viral DNA per filter was used and ^{3}H-RNA came from cells labeled from 17 to 18 hr after infection. In both experiments, the unlabeled adenovirus-infected KB cell RNA was from cells harvested at 18 hr postinfection, whereas the unlabeled adenovirus-infected AGMK cell RNA was from cells harvested at 23 hr postinfection. No inhibition represents hybridization of 2,351 to 2,628 counts per min per filter (0.2 μg of DNA) and 4,781 to 4,826 counts per min per filter (0.5 μg of DNA).*

Transport of adenovirus-specific RNA. To determine whether or not virus-specific RNA moved from its site of synthesis in the nucleus to its locus of translation in the cytoplasm, RNA labeled with ^{3}H-uridine from 28 to 30 hr after infection with either adenovirus or adenovirus and SV40 was extracted from nuclear and cytoplasmic fractions of the infected cells and hybridized with varying amounts of viral DNA. In the experiment described in Fig. 4, 9% of the ^{3}H-uridine incorporated into RNA in abortively infected cells was found in virus-specific sequences. In the cytoplasm, 62.5% of this labeled virus-specific RNA was detected. In doubly infected cells, 14.8% of the ^{3}H-uridine incorporated into RNA was in viral RNA, 52.1% of which was found in the cytoplasm.

Nature of the transported RNA. Cytoplasmic and nuclear fractions were prepared from cells harvested late in the abortive infection of AGMK cells and from late in the productive infection of KB cells; the RNAs extracted from each fraction were used in a hybridization-inhibition experiment with ^{3}H-late RNA prepared from unfractionated adenovirus-infected KB cells. The cytoplasmic and nuclear RNA preparations from abortively infected cells inhibited hybridization to the same extent as did corresponding preparations from infected KB cells (Fig. 5). That is, virus-specific cytoplasmic RNA, whether from abortively infected AGMK cells or from productively infected KB cells, produced about 30% less inhibition than did the nuclear RNAs from the two infected cell types. This correspondence indicates that, in both systems, there is a class of virus-specific sequences, representing about 30% of the total, that does not enter the cytoplasm.

To eliminate the possibility that the difference in the abilities of cytoplasmic and nuclear RNAs to inhibit the hybridization of labeled RNA was simply due to a difference in the molecular size

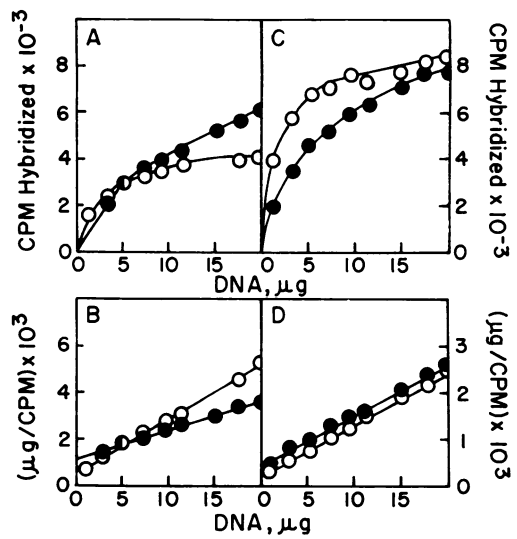

FIG. 4. *Hybridization of adenovirus-specific RNA from the cytoplasm (●) and nucleus (○) of AGMK cells infected either with 2 fluorescent focus-forming units of adenovirus per cell (A) or with both 2 fluorescent focus-forming units of adenovirus per cell and 10 fluorescent focus-forming units of SV40 per cell (C). Monolayer cultures (12.5 × 10⁶ cells) were labeled with 500 μCi of ^{3}H-uridine from 28 to 30 hr after infection. Cells were fractionated by using the Triton X-100 method, and the RNA prepared from each fraction was hybridized with a number of concentrations of viral DNA. The hybridization reaction mixtures contained the following: A, 68,270 counts per min of RNA (●), 80,500 counts per min of RNA (○); C, 64,640 counts per min of RNA (●); 62,340 counts per min of RNA (○) and the quantities of filter-bound viral DNA indicated in the figure. B and D, Reciprocal plots of the data presented in A and C, respectively, with symbols denoting the same experiments.*

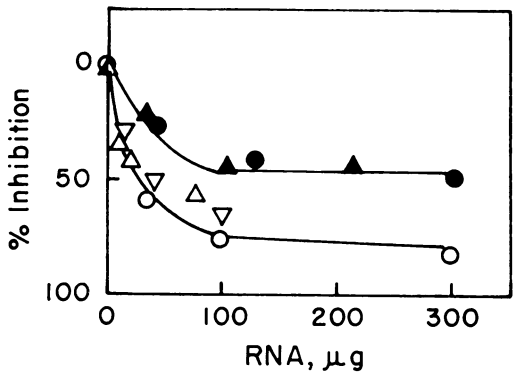

FIG. 5. *Hybridization-inhibition of ^{3}H-late RNA from whole adenovirus-infected KB cells by unlabeled cytoplasmic (●) and nuclear (○) RNAs from infected KB cells or by unlabeled cytoplasmic (▲) and nuclear (△▽) RNAs from infected AGMK cells. The AGMK cells were infected with type 2 adenovirus at a multiplicity of 3 plaque-forming units per cell; KB cells were infected at a multiplicity of 300 plaque-forming units per cell. For the experiment designated ▽, ^{3}H-RNA was prepared from infected KB cells labeled for 17 to 18 hr postinfection with 2.5 μCi of ^{3}H-uridine per ml (0% inhibition represents 1,921 counts per min hybridized per filter). Input of labeled RNA was 188.5 μg (9.5 × 10^5 counts per min) for each reaction mixture. For the experiment designated ●○▲△, ^{3}H-RNA was prepared from cells labeled for 17 to 18 hr postinfection with 3.0 μCi/ml. Input was 61.6 μg (2.8 × 10^5 counts per min) (0% inhibition represents 1,705 to 1,844 counts per min hybridized per filter). Infected AGMK cell RNAs were prepared from cells harvested at 22 hr after infection. Cell fractionation was by the Triton-X100 method. For hybridization, filters contained 0.2 μg of viral DNA.*

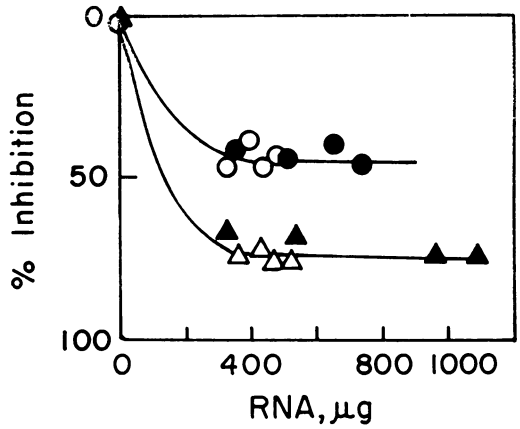

FIG. 6. *Hybridization inhibition of ^{3}H-late RNA prepared from whole infected KB cells by cytoplasmic intact (●) and partially degraded (○) and nuclear intact (▲) and partially degraded (△) RNA. ^{3}H-RNA was prepared from KB cells infected with 100 fluorescent focus-forming units of type 2 adenovirus per cell and labeled with 1 μCi of ^{3}H-uridine per ml from 17 to 18 hr after infection. Nuclear and cytoplasmic RNA was prepared from infected KB cells harvested at 18 hr postinfection. Partial alkaline degradation was accomplished as described in Materials and Methods. A 378-μg amount (1.15 × 10^6 counts per min) of ^{3}H-late RNA and 0.2 μg of viral DNA per filter were used for each reaction mixture.*

of the species prepared from the two cell fractions, the inhibiting RNAs were subjected to a limited alkaline degradation prior to hybridization. As shown previously (15), the conditions used yield a product having a sedimentation coefficient of 4 to 5*S*. The results presented in Fig. 6 show that the cytoplasmic virus-specific RNAs, whether intact or partially degraded, produced less inhibition than did the nuclear preparations.

The possibility that there was a specific loss of a virus-specific RNA species from the cytoplasmic fraction during the extraction of RNA was also considered. RNA was prepared from the cytoplasm of infected KB cells by two procedures. The method of Perry et al. (20), which employs SDS, phenol, and chloroform, reportedly gives quantitative recovery of RNA labeled either with uridine or adenine, whereas there is some loss of adenine-labeled RNA with the procedure employing SDS and hot phenol. However, both preparations produced only partial inhibition of the hybridization of ^{3}H-late RNA prepared from unfractionated adenovirus-infected KB cells (Fig. 7).

DISCUSSION

The limited replication of adenovirus in AGMK cells is enhanced by co-infection with SV40 (2, 8, 17, 18, 24). When 3 plaque-forming units of adenovirus per cell are used, the final yield of adenovirus is about 1,000-fold less than that produced in the double adenovirus-SV40 infection (10). In an attempt to determine the molecular basis for this enhancement, the steps normally occurring in a productive adenovirus infection have been examined.

Despite the fact that only small amounts of adenovirus structural proteins are synthesized in the abortive infection (8, 10, 16), all steps prior to this defect appear normal. That is, early adenovirus proteins are synthesized (8, 10, 16) as well as viral DNA (10, 25, 26) and RNA in amounts comparable to those produced in the enhanced infection (reference 1; this report, Fig. 1). Moreover, as shown in this communication, all species of true late RNA and class II early RNA identified in productive adenovirus in-

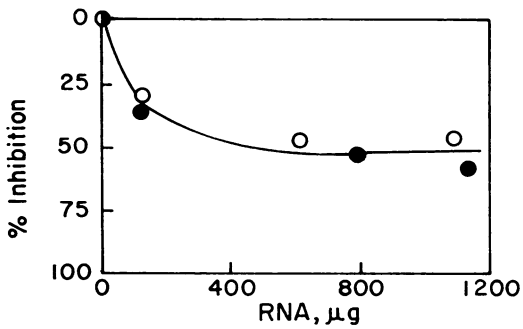

FIG. 7. *Hybridization inhibition of ^{3}H-late RNA from adenovirus-infected KB cells by cytoplasmic RNA extracted from infected KB cells by the SDS-hot phenol method (●) or by the SDS-chloroform-phenol method (○). ^{3}H-RNA was prepared from adenovirus-infected KB cells labeled with 1 μCi of ^{3}H-uridine per ml from 18 to 19 hr after infection. Cytoplasmic RNAs were prepared from infected KB cells harvested at 19 hr after infection. Cells were fractionated by the Triton X-100 method described in Materials and Methods. A 14.7-μg amount (2.1 × 10^5 counts per min) of labeled RNA were used in each reaction mixture.*

fections (15) appear to be synthesized in the abortive infection.

Before translation of adenovirus-specific messenger RNA can occur, it must first be transported to the cytoplasm (29, 31). It was shown by Thomas and Green (30) that, early in a productive adenovirus infection in KB cells, some virus-specific RNA sequences in the nucleus were not present in the cytoplasm. However, such a difference between cytoplasmic and nuclear virus-specific RNA was not observed late in infection. Thus, it was concluded that all adenovirus-specific RNA sequences were eventually present in the cytoplasm. The results presented here (Fig. 5–7) demonstrate a clear difference between the population of RNA sequences found in the nuclear and cytoplasmic fractions late in infection. These experiments differed from those of Thomas and Green in two significant technical aspects. Cells were fractionated by using detergent rather than mechanical lysis of the cell membrane. Also, the sensitive hybridization inhibition technique described by Gillespie (11) was used. It was previously shown that such a technique is required for an accurate study of transcription in adenovirus-infected cells (15). That caution is needed in the use of hybridization competition and inhibition techniques when analyzing the complex mixtures of RNA obtained from mammalian cells has been emphasized both by Darnell (7) and by Pettijohn and their associates (4, 21, 22, 28). Moreover, the observation that there is a class of virus-specific RNA confined to the nucleus of KB cells infected with type 5 adenovirus was recently reported by Madore and Bello (Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 216, 1972).

It has been observed that the electrophoretic mobilities of adenovirus-specific RNA species prepared from nuclei of infected KB cells are generally much less than those of cytoplasmic species (reference 19; J. J. Lucas and H. S. Ginsberg, *unpublished results*). Assuming that this difference is due neither to a preferential degradation of cytoplasmic species during extraction procedures nor to the binding of contaminating molecules which would hinder the mobility of the nuclear species, it may be concluded that the sizes of virus-specific RNA in the nucleus are much greater than those in the cytoplasm. To eliminate the possibility that the difference in the behavior of cytoplasmic and nuclear RNA in hybridization-inhibition was due to this size difference, experiments were performed with RNA which had been reduced to a standard size of 4 to 5*S* by controlled alkaline degradation. Identical results were then obtained with both intact and partially degraded preparations (Fig. 6).

Moreover, a selective loss of RNA species during the extraction of cytoplasmic RNA with SDS and hot phenol does not explain the 30% fewer RNA species found as compared to nuclear RNAs. Extraction with SDS, phenol, and chloroform, which permits a quantitative recovery of both uridine- and adenine-labeled RNA molecules (20), also yielded cytoplasmic RNA that gave only partial inhibition of the hybridization of labeled RNA prepared from unfractionated infected cells (Fig. 7).

These findings indicate that the transport of adenovirus-specific RNA is nonconservative since all RNA sequences synthesized in the nucleus do not enter the cytoplasm. The observations that at least some cytoplasmic virus-specific species may be cleavage products of larger precursor molecules in the nucleus (19) and that the RNAs contain adenine-rich regions (23) suggest that the processing and transport of adenovirus-specific RNA may be similar in many ways to that of mammalian cell RNA species. But the function of the adenovirus-coded sequences confined to the nucleus is still unknown.

The mechanism by which SV40 enhances the replication of adenovirus in AGMK cells remains unclear. Two possible explanations, however, are being considered. The technique of DNA-RNA hybridization permits the detection

of RNA sequences as small as 10 to 20 nucleotides in length (32), and therefore it is possible that the hybridizable adenovirus-specific RNA is actually present as nonfunctional small pieces, owing to an endonucleolytic activity present in monkey cells. Co-infection with SV40 might protect the RNA from this activity, for example, by causing a repression of the enzyme. Determination of the molecular weights of adenovirus-specific RNA in productive and abortive infections by using the technique of polyacrylamide gel electrophoresis is in progress. Alternatively, the defect preventing efficient replication of adenoviruses may be directly at the level of translation. In this case, SV40 would provide a function which permits the translation of adenovirus messenger RNAs. Further study of this complex process will be greatly aided by the recently developed systems for in vitro synthesis of adenovirus-specific proteins (6, 35).

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LITERATURE CITED

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