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Biochemical Consequences of Type 2 Adenovirus and Simian Virus 40 Double Infections of African Green Monkey Kidney Cells

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African green monkey kidney (AGMK) cells were nonpermissive hosts for type 2 adenovirus although the restriction was not complete; when only 3 plaque-forming units/cell was employed as the inoculum, the viral yield was about 0.1% of the maximum virus produced when simian virus 40 (SV40) enhanced adenovirus multiplication. The viral yield of cells infected only with type 2 adenovirus increased as the multiplicity of infection was increased. Type 2 adenovirus could infect almost all AGMK cells in culture; adenovirus-specific early proteins and DNA were synthesized in most cells, but small amounts of late proteins were made in relatively few cells. Even when cells were infected with both SV40 and adenovirus, only about 50% were permissive for synthesis of adenovirus capsid proteins. Approximately the same quantity of adenovirus deoxyribonucleic acid (DNA) was synthesized in the restricted as in the SV40-enhanced infection. However, in cells infected with SV40 and type 2 adenovirus, replication of SV40 DNA was blocked, multiplication of SV40 was accordingly inhibited, and synthesis of host DNA was not stimulated. To enhance propagation of type 2 adenovirus, synthesis of an early SV40 protein was essential; 50 μg of cycloheximide per ml prevented the SV40-induced enhancement of adenovirus multiplication, whereas 5×10^{-6} M 5-fluoro-2-deoxyuridine did not abrogate the enhancing phenomenon.

Rabson et al. reported that simian virus 40 (SV_{40}) facilitated the replication of types 5 and 12 adenovirus in African green monkey kidney (AGMK) cells which are normally restrictive for the multiplication of human adenoviruses (32). Enhancement of adenovirus replication was dependent on prior or simultaneous infection of the cells with SV_{40} (28, 32). Other investigators (34) extended the list of human adenoviruses whose propagation SV_{40} enhanced in AGMK cells, making it appear likely that most, if not all, human adenoviruses could engage in this functional interaction with an unrelated virus. Enhancement was not found when other deoxyribonucleic acid (DNA) viruses were substituted for SV_{40} (11, 28).

Subsequent studies showed that human adenoviruses undergo an abortive replicative cycle in AGMK cells (11). Under conditions of single infection, early adenovirus antigens could be detected by immunofluorescence (11, 25) from which it became apparent that the initial stages of the replicative cycle, involving adsorption, penetration, and uncoating of the infecting virions, are unaffected. Analyses of DNA extracted from singly and doubly infected cultures revealed that adenovirus DNA is synthesized during abortive infections (33, 35). Similarly, Baum et al. (2) extracted adenovirus-specific ribonucleic acid (mRNA) from singly and doubly infected cells, but the nature of the mRNA detected by hybridization (i.e., early or late) was not investigated.

Under the conditions described, adenoviruses in some respects operationally resemble a conditionally lethal or defective mutant in which the AGMK cell is a nonpermissive host. SV_{40}, a genetically unrelated virus, serves a helper function which permits multiplication of the otherwise "defective" virus. In the absence of well-characterized adenovirus mutants, it seemed possible that adenoviruses in AGMK cells

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might serve as a substitute that could help reveal some of the factors which regulate the sequential synthesis of adenovirus gene products. The present report describes experiments designed to investigate further the nature of the restriction imposed on propagation of human adenoviruses in AGMK cells and to assess the role SV_{40} plays in converting the abortive infection into a productive one.

MATERIALS AND METHODS

Viruses. The prototype strain of type 2 adenovirus, which was used in the experiments to be described, was plaque-purified three times and shown to be free from adeno-associated virus (AAV) by complement-fixation and electron microscopic techniques. KB cell spinner cultures were infected to prepare pools of adenovirus as previously described (23). The SV_{40} virus (kindly supplied by A. Girardi, Wistar Institute) was also plaque-purified three times in CV-1 cells. SV_{40} was prepared in roller-bottle cultures of CV-1 cells infected with 0.1 plaque-forming unit (PFU)/cell and harvested when the cell sheet revealed 2 + cytopathic effects. The cell suspension was concentrated 10- to 20-fold and sonically treated (Measuring & Scientific Co. Ltd., ultrasonic disintegrator) for 1 min.

Tissue culture. KB cells were propagated in spinner cultures employing minimal essential medium (MEM) supplemented with 10% calf serum. Monolayers of KB cells were also prepared in 35-mm plastic petri dishes with the same medium; for infection of monolayer cultures, MEM supplemented with 5% calf serum was used (10).

Primary AGMK cells were purchased from Flow Laboratories, Rockville, Md., and grown in basal Eagle medium containing 5% fetal calf serum.

Infectivity assay. Adenovirus was assayed on monolayers of KB cells by the plaque assay previously described (23). SV_{40} was assayed in monolayers of AGMK cells by using the same procedure, except that 15% fetal calf serum was present in the overlay. The fluorescent focus assay of Philipson (30) revised by Thiel and Smith (39) was also employed for titration of adenovirus. The Zeiss G.F.L. microscope illuminated by an Osram HBO 200-w mercury lamp was fitted with a 5-mm grid in one ocular to facilitate focus counting. Fluorescein-conjugated goat anti-rabbit gamma globulin (Hyland Laboratories, Los Angeles, Calif.) and rhodamine bovine albumin (Microbiological Associates, Bethesda, Md.) were employed in the indirect technique.

Immunofluorescence of "early" viral antigens. AGMK cells, grown to confluence on cover slips (9 by 22 mm) were infected with the appropriate virus or viruses. To insure that late adenovirus antigens were not produced, 5-fluoro-2-deoxyuridine (FUdR; Hoffman-LaRoche, Nutley, N.J.), at a final concentration of 5×10^{-6} M, was added to the medium after viral adsorption and again 24 hr after infection. The cover slips were rinsed in 0.15 M NaCl buffered with 0.01 M phosphate [*p*H 7.2 (PBS)] 36 or 48 hr postinfection and fixed in cold acetone at −20 C for 10 min. The SV_{40} virus-infected cells were stained with fluorescein-tagged pooled gamma globulin from hamsters bearing SV_{40}-induced tumors (Flow Laboratories, Inc.). Specific rabbit antisera against early or late adenovirus antigen(s) (14, 36) were used in the indirect fluorescent-antibody procedure. All specimens, including uninfected control slides, were examined under an oil immersion apochromatic objective (40 ×).

Complement fixation. A microtechnique was employed (37). Antigen titers are expressed as the highest dilution that completely fixed 2 units of guinea pig complement in the presence of 4 to 8 units of antibody.

Preparation of antisera. Rabbits were immunized with purified type 5 adenovirus hexon (24), purified type 2 adenovirus, or a homogenate prepared from cells infected with type 2 adenovirus in the presence of 5×10^{-5} M FUdR and harvested 16 hr after infection. This latter inoculum was tested for the presence of infectious virus, and, after immunization, the antisera obtained were examined for the presence of antibody against late viral proteins. In each instance, the immunizing material was mixed with Freund's adjuvant and injected in 0.5-ml quantities into the four foot pads. One month later, each rabbit received 0.5 ml subcutaneously in the inguinal region and was bled 10 days afterwards.

Autoradiography. Confluent cultures of AGMK cells on cover slips were infected with virus; others were mock-infected and served as controls. Eighteen hours after infection, 0.2 μc of ^{3}H-thymidine per ml (thymidine-*methyl*-^3H, 3.6 mc/mg, New England Nuclear Corp., Boston, Mass.) was added to the maintenance medium. The medium was removed 30 hr after infection, and the cell sheets were washed three times with PBS and fixed in ethanol-glacial acetic acid (3:1, v/v) for 10 min at room temperature. The cultures were washed successively in 70, 40, 20, and 10% aqueous ethanol washes, rinsed in distilled water, and extracted twice with cold 5% trichloroacetic acid for 10 min (1). The cultures were then rinsed in distilled water, 70% ethanol, and 95% ethanol and allowed to air-dry before being mounted on microscope slides. The fixed cultures were dipped in 44% NTB2 emulsion (Kodak), dried for 1 hr, and stored at 4 C for 7 days. After development, the slides were stained with hematoxylin and eosin and each was studied microscopically to determine the proportion of cells containing isotope and the number of grains per nucleus. A minimum of 1,000 cells were counted per preparation. Uninfected cells to which ^{3}H-thymidine had not been added were examined as emulsion control preparations; less than three grains per cell were found.

Preparation of ^{3}H-labeled SV_{40} DNA. To each confluent culture of CV-1 cells in production roller bottles, SV_{40} at a multiplicity of approximately 0.1 PFU/cell was added in 10 ml of infecting fluid. After 2 hr at 37 C, 200 ml of maintenance medium was added to each culture. ^{3}H-thymidine (0.5 μc per ml of medium) was added 24 hr postinfection. At 6 to 7 days after infection, when greater than 50% of the cells showed cytopathic effects, the cells were scraped

into the medium and sedimented by centrifugation. Virus was purified by the procedure of Black et al. (5) by employing two equilibrium density gradient centrifugations in CsCl. After dialysis against tris(hydroxymethyl)aminomethane (Tris)-buffered saline (Tris-hydrochloride, 0.01 M, *p*H 8.1; NaCl, 0.15 M), the viral DNA was extracted by the method of Watson and Littlefield (42). The DNA obtained had a specific activity of 6,250 counts per min per μg of DNA, consisted of a single 20*S* peak after band centrifugation in CsCl (41), and was eluted in a single peak from a methylated albumin-kieselguhr (MAK) column (26) at 0.55 M NaCl.

Purification of adenovirus and extraction of viral DNA. After preliminary preparation, virus was purified by centrifugation in CsCl gradients and the DNA was obtained as previously described (4, 24).

Preparation of labeled DNA from virus-infected and uninfected cells. AGMK cells were grown to confluence in roller bottles; some cultures were not infected, and the remaining ones were infected with adenovirus or adenovirus and SV_{40} virus. Sixteen hours after infection, ^{3}H-thymidine (1 μc per ml of medium) was added to each bottle; the cultures were incubated for 20 hr after which the cells were harvested and washed. DNA was extracted by a procedure similar to that employed by Reich et al. (35). Approximately 10^8 cells were suspended in 2 ml of a buffer containing 0.1 M NaCl, 0.01 M ethylenediaminetetraacetic acid (EDTA), 0.05 M Tris-hydrochloride (*p*H 8.6), and 0.5% sodium dodecyl sulfate (SDS). Freshly prepared Pronase (Calbiochem, grade B, 45,000 PUK/g) was added to give a final concentration of 1 mg/ml, and the suspensions were incubated at 37 C with intermittent gentle shaking for 2 to 3 hr or overnight at room temperature. After two to three extractions with 80% freshly distilled phenol, the DNA solution was extracted once with anhydrous ether and dialyzed against 0.1 SSC (0.15 M NaCl plus 0.015 M sodium citrate) at 4 C for 24 to 48 hr, with at least three changes of buffer. A few drops of chloroform were added to the DNA and it was stored at 4 C. This procedure recovered approximately 90% of the DNA.

Selective extraction of viral DNA. Confluent monolayer cultures of AGMK cells in 60-mm plastic petri dishes were infected with adenovirus, SV_{40} virus, or combinations of both viruses. In each experiment, cultures were also mock-infected. Three cultures were employed for each group. After infection, spent medium (from 1- to 2-day-old cultures) was added. After 16 hr of incubation at 37 C in a CO_2 incubator, 10 μc of ^{3}H-thymidine or ^{14}C-thymidine was added to each plate. Thirty-six hours after infection, the medium was discarded and the cell sheets were washed once with PBS. A 1-ml amount of 0.6% SDS in 0.01 M EDTA, 0.01 M Tris-hydrochloride (*p*H 8.1) was added to each plate, and the viral DNA was extracted by the method of Hirt (20) for the separation of polyoma virus DNA from host nuclear DNA. To determine the proportion of adenovirus or SV_{40} virus DNA not extracted in the selective procedure, the pellets were treated with Pronase and the DNA was extracted three times with chloroform-isoamyl alcohol (24:1) as in the procedure of Marmur (27) or with 80% phenol. The types of DNA were analyzed by isopycnic gradient centrifugation in CsCl. The selective extraction procedure was found to recover 95% of the adenovirus and SV_{40} DNA from infected cells.

To determine the quantity of host DNA extracted in the selective procedure described above, growing cells were labeled with ^{3}H-thymidine until confluent monolayers were obtained. Cold medium was substituted for the radioactive medium for 24 hr to deplete the ^{3}H-thymidine cellular pool. The cultures were subsequently infected and labeled with ^{14}C-thymidine; 36 hr after infection, the DNA was extracted as described above. A portion of each extract was subjected to rate zonal centrifugation, and the ^{3}H and ^{14}C content of each fraction in the gradient was determined. No significant level of ^{3}H counts was observed.

Sedimentation analysis (band centrifugation) of DNA. A 10- to 20-μg amount of DNA in 0.2 ml was layered onto 3 ml of CsCl (Harshaw Chemical Co., optical grade) having a density of 1.5 g/ml in 0.01 M EDTA, 0.01 M Tris-hydrochloride (*p*H 8.1) and was centrifuged in a Spinco SW 39 rotor at 99,972 $\times$ *g* for 3.25 hr at 20 C (9, 41). Fractions (two drops) were collected from the bottom of the tubes onto Whatman no. 3 filter-paper discs (2.3 cm). The discs were briefly oven-dried, washed twice with 5% ice-cold trichloroacetic acid for 20 min, washed with acetone, and dried. Radioactivity was assayed in a Packard Tri-Carb liquid scintillation spectrometer using 5 ml of phosphor (40).

Chromatography on MAK columns. MAK was prepared as described by Mandell and Hershey (26). The column (1.9 by 6 cm) was made in a single layer (38), and the adsorbed DNA was eluted in steps with increasing concentrations of NaCl (0.5 to 0.7 M) containing 0.05 M phosphate buffer (*p*H 6.7). Fractions of 5 ml were collected and 0.1 ml of each was spotted on a filter-paper disc for assay in a scintillation spectrometer (40). DNA was also measured by optical density at 260 nm and by the diphenylamine reaction (6).

Isopycnic gradient centrifugation. DNA samples were layered on 4 ml of CsCl solution having a density of 1.7 g/ml in Tris-hydrochloride-EDTA buffer (*p*H 8.1) and were centrifuged in the Spinco SW 39 rotor for 70 hr at 99,972 $\times$ *g* at 20 C. Fractions were collected and counted as described above. Fractions at both sides of the peaks were collected under oil for estimation of buoyant density by using an Abbe refractometer.

RESULTS

Multiplication of type 2 adenovirus in AGMK cells in the absence or presence of SV_{40} virus. Adenoviruses reportedly do not multiply in primary monkey kidney cells except in the presence of SV_{40} virus (11, 28, 32). The experiments that described this enhancement phenomenon were carried out with multiplicities of adenovirus which presumably infected less than one-half of the cells (11, 28, 32). However, to investigate biochemically the mechanism by

which SV_{40} converts an abortive adenovirus infection into a productive infection, it was desirable to infect all of the cells. Preliminary experiments in which various multiplicities of each virus were employed demonstrated that 30 PFU of SV_{40} per cell produced optimum enhancement of adenovirus multiplication, that maximum enhancement of adenovirus propagation could be detected when a relatively low multiplicity of type 2 adenovirus was employed, and that some adenovirus multiplication ensued, albeit a relatively small amount, when cells were infected with adenovirus alone. The larger the adenovirus inoculum, the greater the amount of adenovirus produced.

An analysis of the multiplication of type 2 adenovirus in AGMK cells in the presence and absence of SV_{40} is summarized in Fig. 1. The yield of adenovirus was enhanced approximately 1,000-fold when multiplicities of 3 PFU of adenovirus per cell and 30 PFU of SV_{40} per cell were added to the cultures. When 30 PFU of adenovirus per cell was used, the viral yield was increased only about 250 times. Although adenovirus propagation was greatly limited under conditions of single infection, some viral production occurred. Moreover, the eclipse period of adenovirus multiplication was similar, approximately 16 hr, whether cells were infected with type 2 adenovirus alone or whether SV_{40} was added to the inoculum.

In addition to assays for infectious virus in the experiments described above, complement-fixation titrations were carried out to compare the

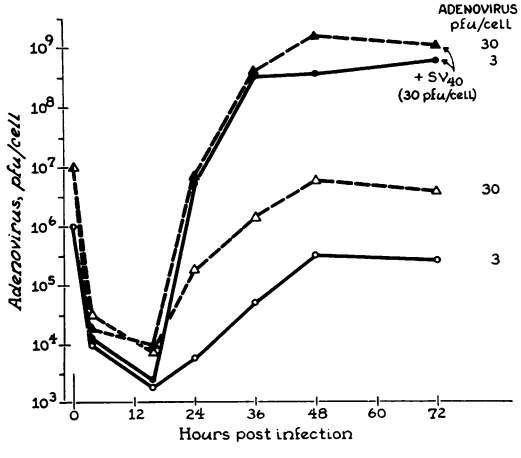

FIG. 1. *Multiplication of type 2 adenovirus in AGMK cells. Monolayers of AGMK cells were infected with adenovirus or adenovirus plus SV_{40}. Replicate cultures were harvested at the times indicated. The washed cell pellets were sonically treated and assayed for adenovirus.*

TABLE 1. *Production of adenovirus hexon antigen in AGMK cells infected with type 2 adenovirus alone or infected with SV40 and type 2 adenovirus*

Infected with[a]		Complement-fixation titer[b]			
Adenovirus	SV40	4[c]	24	36	48
PFU/cell	*PFU/cell*				
3		0[d]	4	8	16
3	30	0	16	128	256
30		0	16	16	32
30	30	0	64	128	256
	30	0	0	0	0
		0	0	0	0

[a] Duplicate cultures of AGMK cells were infected. Cells were harvested, washed with PBS, and sonically treated for 1 min.

[b] Reciprocal of the complement-fixation titer. Antibody to purified hexon was employed for the titration.

[c] Hours after infection.

[d] Titer of <1:2.

relative amounts of adenovirus capsid proteins made in the restrictive and productive infections. These titrations were done with a serum from rabbits immunized with purified hexon (24) to permit detection of relatively small amounts of viral antigens, because complement fixation did not occur with uninfected cell antigens when this serum was employed. The results of a representative experiment (Table 1) demonstrate that adenovirus hexon protein (17) was made in limited quantities when cells were infected only with type 2 adenovirus, and that SV_{40} infection markedly increased the synthesis of adenovirus capsid protein.

Immunofluorescence assay of cells synthesizing adenovirus early proteins and capsid antigens. Cultures infected with type 2 adenovirus produce relatively small numbers of infectious particles; the amount of virus produced is greatly increased (Fig. 1) when cultures are also infected previously or simultaneously with SV_{40} (25, 28, 32). To understand this phenomenon, it was necessary to determine how many cells were infected when adenovirus alone was added and whether, under conditions of enhanced double infections, more cells synthesized early as well as late (i.e., capsid antigen) proteins. To answer the first query, cells were infected in the presence of 5×10^{-6} M FUdR, and 16 hr later they were examined by using serum from rabbits immunized with KB cells infected with type 2 adenovirus in the presence of FUdR. With a small inoculum of adenovirus alone (i.e., input multiplicity of 3 PFU/cell), early protein could be detected in

TABLE 2. *Detection of early adenovirus antigens*[a] *by immunofluorescence*

Infected with		Per cent cells positive[b]
Adenovirus	SV40	
PFU/cell	*PFU/cell*	
3		30
3	30	85–90
30		85–90
30	30	95
	30	0
		0

[a] AGMK cells infected in the presence of 5×10^{-6} M FUdR.

[b] Slides were stained by the indirect method by using rabbit serum containing antibodies to early adenovirus antigens and anti-rabbit goat gamma globulin conjugated with fluorescein isothiocyanate.

TABLE 3. *Synthesis of early adenovirus antigens*

Infected with		Complement-fixation titer[b]			
Adenovirus[a]	SV40[a]	4[c]	24	36	48
PFU/cell	*PFU/cell*				
3		0[d]	0	4	4
3	30	0	0	16	16
30		0	0	8	8
30	30	0	8	32	32
	30	0	0	2	2
		0	0	2	2

[a] Infected in the presence of 5×10^{-6} M FUdR.

[b] Complement-fixation test performed using rabbit serum containing antibodies only to early adenovirus antigens. Titer is expressed as the reciprocal. Purified hexon from type 2 adenovirus infected cells did not react with the serum employed.

[c] Hours after infection.

[d] Titer of <1:2.

approximately one-third of the cells (Table 2), but the addition of SV_{40} to the inoculum increased the number of cells showing antigen as well as the intensity of the antigen. Hence, adenovirus could infect almost all of the cells. Although early proteins were detected in only about 30% of the cells when adenovirus alone was used, it is likely that almost all of the cells produced some early proteins and that the addition of SV_{40} either quantitatively or qualitatively altered the synthesis of early proteins so that the immunofluorescence technique easily detected them. Assay of early proteins by complement-fixation assays substantiated the impression that more adenovirus-specific early proteins were made in cells infected with both adenovirus and SV_{40} than when only adenovirus was used as the infecting inoculum (Table 3). It could not be determined whether unique early proteins were made only in the cells infected with both viruses.

Cells were similarly infected in the absence of FUdR to determine in the abortive infection whether each cell was making a small amount of capsid protein or whether only a rare cell was productively infected. It was surprising that 20 to 30% of the cells produced capsid antigens, depending upon the multiplicity of adenovirus infection (Table 4), that the number of cells was only doubled under conditions in which the yield of infectious virus was enhanced 1,000-fold (Fig. 1), and that in cells infected with both viruses the quantity of hexon antigen was only increased about 16-fold (Table 1). Thus, although all cells could be infected with adenovirus, SV_{40} could convert a partially abortive infection into a productive infection in about 50% of the cells; the remainder of the cells were still apparently nonpermissive for propagation of infectious adenovirus.

DNA synthesis in cells infected with type 2 adenovirus or adenovirus and SV_{40}. Since adenovirus can infect all AGMK cells in a culture and adenovirus-specific early proteins can be made

TABLE 4. *Detection of adenovirus capsid antigens by immunofluorescence*[a]

Infected with		Per cent cells positive[b]
Adenovirus	SV40	
PFU/cell	*PFU/cell*	
3		22
		20
		20
		23
3	30	39
		39
		41
		42
30		24.5
		31.5
30	30	50
		49
		45
		57

[a] Indirect method with serum from rabbits immunized with purified type 2 adenovirus.

[b] Examined 36 hr after infection.

in the infected cells, it was necessary to determine whether the restriction in synthesis of capsid proteins resulted from a failure to replicate viral DNA in the nonpermissive cells (12) or whether some other biosynthetic step was blocked. To accomplish this objective, DNA synthesis in AGMK cells infected with various multiplicities of adenovirus in the absence or presence of SV_{40} was studied in several ways: (i) incorporation of ^{3}H-thymidine to measure the rate of synthesis of total DNA, (ii) autoradiography to enumerate the number of cells synthesizing DNA, (iii) separation of adenovirus and SV_{40} DNA by velocity centrifugation in CsCl gradients, and (iv) separation of viral from host DNA by chromatography on MAK columns.

Rate of DNA synthesis. At intervals, ^{3}H-thymidine (2 μc/ml) was added for 1 hr to uninfected cells and to cells infected with type 2 adenovirus (3 PFU/cell), with adenovirus (3 PFU/cell) and SV_{40} (30 PFU/cell), or with SV_{40} (30 PFU/cell). The data obtained (Fig. 2) demonstrate that DNA synthesis was markedly increased under each of the conditions of infection, that the rates of DNA synthesis were similar in cells abortively infected with adenovirus or productively infected when the helper SV_{40} was added, and that infection with SV_{40} alone stimulated DNA synthesis approximately three times more (13, 19, 22) than did adenovirus and SV_{40} together. It should also be noticed that, whereas DNA synthesis attained a maximum rate at 15 to 18 hr after infection with adenovirus or adenovirus and SV_{40}, maximum synthesis occurred approximately 30 hr after SV_{40} infection. This experiment implied that some event in the multiplication of type 2 adenovirus blocked replication of SV_{40} DNA or host DNA, which is enhanced in cells infected only with SV_{40} (13, 19, 22), or that it inhibited the synthesis of both species of DNA. The following analyses were done to clarify this phenomenon.

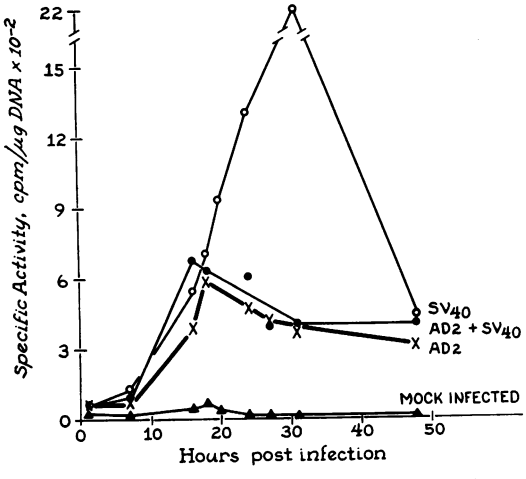

FIG. 2. *Rate of DNA synthesis in AGMK cells infected with type 2 adenovirus or with type 2 adenovirus plus SV_{40}. Confluent monolayers of AGMK cells were infected with type 2 adenovirus (3 PFU/cell), type 2 adenovirus (3 PFU/cell) plus SV_{40} (30 PFU/cell), or SV_{40} alone (30 PFU/cell). Uninfected cultures were studied. At various times after infection, some of the cultures were pulse-labeled with ^{3}H-thymidine (2 μc/ml) for 1 hr and then harvested and washed. The cell pellets were treated with 5% trichloroacetic acid at 4 C. The precipitate was assayed for total DNA and incorporation of ^{3}H-thymidine.*

Separation of adenovirus and SV_{40} DNA by velocity (band) sedimentation in CsCl. Centrifugation (41) of an artificial mixture of type 2 adenovirus DNA and SV_{40} virus DNA extracted from purified viruses sharply separated the two species of viral DNA (Fig. 3). DNA species selectively extracted (20) from cells infected with either virus alone or with both viruses had sedimentation characteristics similar to those of DNA species from purified viruses; conveniently host DNA was not present in the extracted samples. The results of a representative experiment (Fig. 4) demonstrate that (i) synthesis of SV_{40} DNA was increasingly inhibited as the multiplicity of adenovirus infection was increased, (ii) replication of similar quantities of adenovirus DNA occurred in restricted and enhanced infections, and (iii) replication of adenovirus DNA was increased with increasing multiplicities of infecting adenovirus.

Separation of host and viral DNA on MAK

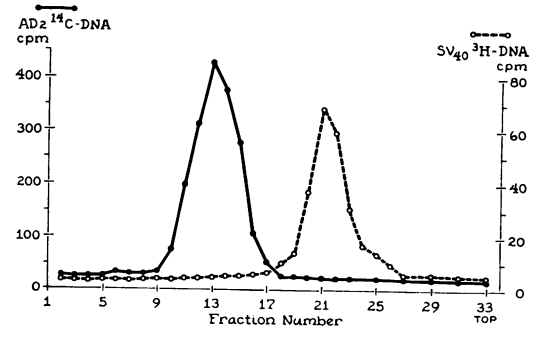

FIG. 3. *Sedimentation analysis of an artificial mixture of type 2 adenovirus ^{14}C-DNA and SV_{40} ^{3}H-DNA. The two species of DNA (10 μg of each in 0.2 ml) were layered onto 3 ml of CsCl (ρ = 1.5 g/ml) and centrifuged at 99,972 × g for 3.25 hr at 20 C in an SW 39 rotor. Two-drop fractions were collected from the bottom of the tube.*

columns. Undenatured SV_{40} and type 2 adenovirus DNA could not be distinguished by chromatography on MAK columns under the conditions employed. Nevertheless, this procedure distinctly separated viral from host-cell DNA, as shown by chromatography of an artificial mixture of type 2 adenovirus and monkey kidney cell DNA (Fig. 5); greater than 90% of the DNA was recovered. It was therefore possible to determine whether adenovirus infection alone or infection in partnership with SV_{40} blocked synthesis of host-cell DNA, and whether the quantity of DNA made in the restricted infection was proportionate to the amount of infectious virus propagated in the enhanced adenovirus-SV_{40} infection. The data obtained from an experiment in which cells were infected only with type 2 adenovirus (Fig. 6) bear upon two of these points: (i) replication of host-cell DNA was blocked and (ii) the quantity of adenovirus DNA synthesized was greatly in excess of either the quantity of virus made in the single infection or the 1,000-fold more virus produced in an enhanced infection. A total of 1.2×10^6 PFU was synthesized in the infected AGMK cells, whereas 72 μg of viral DNA was recovered in the 0.55 M NaCl fractions. If DNA is 13% of the weight of the virion and the virion weighs 2×10^{-16} g (18), then 3.12×10^{-3} μg of DNA was contained in the infectious particles propagated. Even if the assembly of adenovirus in AGMK cells is similar to that in HeLa cells [i.e., only about 10% of the DNA synthesized is incorporated into virions (16)], a very large excess of viral DNA was replicated in the restricted infection as compared to the number of virions assembled.

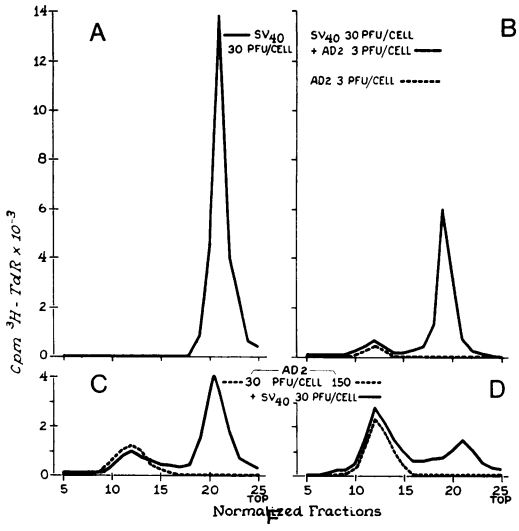

FIG. 4. *Biosynthesis of type 2 adenovirus and SV_{40} DNA in AGMK cells infected with one or both viruses. Three replicate monolayer cultures of AGMK cells were infected with each inoculum: SV_{40}, 30 PFU/cell (A, B, C, D); type 2 adenovirus, 3 PFU/cell (B), 30 PFU/cell (C), or 150 PFU/cell (D). "Spent" medium obtained from the uninfected cultures was added to the cultures after infection. Sixteen hours after infection, 10 μc of ^{3}H-thymidine or ^{14}C-thymidine was added to each culture; 36 hr postinfection the cell sheets were washed three times and viral DNA was selectively extracted. Sedimentation analyses were performed as described in Fig. 3.*

A similar chromatographic separation was also made on the total DNA extracted from AGMK cells infected with both type 2 adenovirus (10 PFU/cells) and SV_{40} (30 PFU/cell). The data from one such experiment (Fig. 7) demonstrate that biosynthesis of host-cell DNA is not stimulated as it is in cells infected only with SV_{40} (19, 22). A small amount of DNA was eluted in the first fraction of 0.625 M NaCl, but its distribution was not characteristic of normal host-cell DNA. This DNA was not further identified. When DNA eluted from MAK at 0.55 M NaCl was separated by velocity sedimentation in CsCl, it was shown to consist predominantly of adenovirus DNA. Hence, adenovirus infection not only blocked replication of its helper DNA, SV_{40}, but also suppressed the stimulated synthesis of AGMK cell DNA.

Number of cells synthesizing DNA when singly or doubly infected. Autoradiographic techniques were used to determine the number of cells which synthesized DNA under the conditions of infection employed. The data summarized in Fig. 8 reveal that SV_{40} (30 PFU/cell) induced almost all cells to synthesize DNA but that replication of DNA occurred in only about 30% of the cells infected with type 2 adenovirus at a low multiplicity of infection (i.e., 3 PFU/cell), approximately the same number in which production of specific early proteins could be detected. It is striking that infection with 3 PFU of adenovirus per cell and 30 PFU of SV_{40} per cell only increased slightly the number of cells synthesizing DNA as compared to adenovirus alone, although infection with only SV_{40} initiated DNA biosynthesis in practically all of the cells. An inoculum of type 2 adenovirus of 30 or 150 PFU/cell, with or without SV_{40}, caused replication of DNA in almost all of the cells. The experiments also confirmed the evidence presented above (Fig. 2 and 4) that more DNA was made in cells infected only with SV_{40} than in cells doubly infected with SV_{40} and adenovirus except at a very high adenovirus multiplicity (i.e., 150 PFU/cell).

Multiplication of SV_{40} in cells infected with

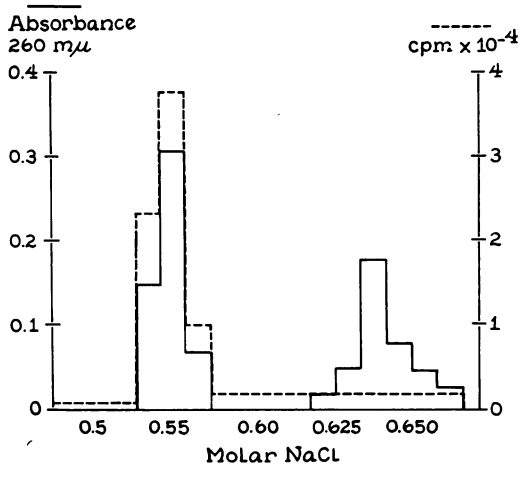

FIG. 5. *Chromatographic separation of an artificial mixture of ^{14}C-labeled type 2 adenovirus DNA and unlabeled, normal monkey kidney cell DNA on a MAK column. Type 2 adenovirus DNA was obtained from purified virus. Approximately 50 μg of each DNA was layered onto the column (1.9 by 6 cm) in 0.2 M NaCl containing 0.05 M phosphate buffer, pH 6.7. Elution was carried out with 0.05 M stepwise increases in NaCl concentration; 5-ml fractions were collected. DNA recovery from the column was greater than 90%.*

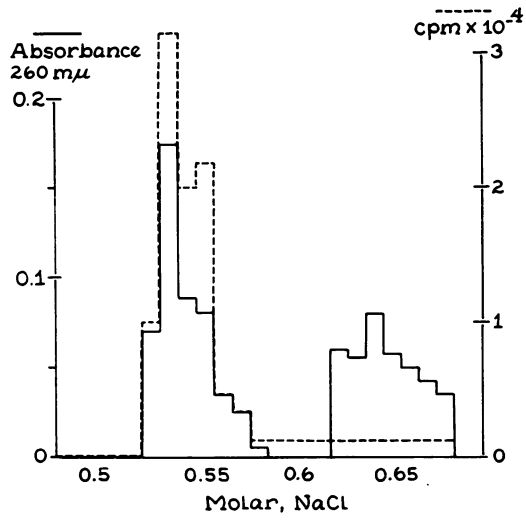

FIG. 6. *Chromatographic separation on MAK column of DNA extracted from AGMK cells infected with type 2 adenovirus. Confluent AGMK cells were infected with type 2 adenovirus, 30 PFU/cell. ^3H-thymidine (1 μc/ml) was added 16 hr after infection; the cells were incubated an additional 20 hr and harvested. Cells were disrupted with 0.5% SDS in 0.1 M NaCl, 0.01 M EDTA, and 0.05 M Tris-hydrochloride buffer at pH 8.6, and DNA was extracted as described in Material and Methods. The resultant DNA extract was dialyzed against three changes of 0.2 M NaCl, 0.05 M phosphate buffer (pH 6.7) at 4 C for 48 hr. A 50-μg amount of DNA was added to the MAK column and elution was accomplished as described in Fig. 5. Recovery of DNA was greater than 90%.*

type 2 adenovirus. Biosyntheses of SV_{40} (7) and cellular DNA were markedly decreased in cells concomitantly infected with type 2 adenovirus. It was therefore predicted that production of SV_{40} virions would also be suppressed in cells infected with adenovirus. The results of experiments to test this prediction (Table 5) demonstrate that propagation of SV_{40} was indeed inhibited under conditions in which the multiplication of adenovirus was enhanced. These studies also showed that purified type 2 adenovirus similarly reduced the multiplication of SV_{40} and that crude virus inoculum did not contain sufficient soluble adenovirus antigens (24) to inhibit SV_{40} propagation. In similar experiments, it was also found that greater than 95% of the cells synthesized early SV_{40} T antigen (31), whether infected only with SV_{40} or with both viruses.

Effect of inhibitors of macromolecular synthesis on capacity of SV_{40} to enhance multiplication of type 2 adenovirus. Since type 2 adenovirus inhibited synthesis of SV_{40} DNA and late proteins, although multiplication of adenovirus was enhanced, it seemed probable that an early event in the biosynthesis of SV_{40} was responsible for the increased adenovirus propagation. This possibility was tested by utilizing the finding that the rate of adenovirus multiplication was distinctly greater in cells infected with SV_{40} 12 to 16 hr before infection with adenovirus than when SV_{40} and adenovirus were added simultaneously (Fig. 9 and 10). Early viral proteins are synthesized in the absence of SV_{40} DNA synthesis (11); it was therefore possible to distinguish whether biosynthesis of SV_{40}-induced early proteins or SV_{40} DNA was necessary to increase adenovirus multiplication in AGMK cells. At the time of infection with SV_{40} (30 PFU/cell), 5×10^{-6} M FUdR was added to stop DNA synthesis. Sixteen hours later, cultures were infected with type 2 adenovirus and excess thymidine (5×10^{-5} M) was added to permit DNA synthesis. The results of a representative experiment (Fig. 9) demonstrate that (i) cells infected with SV_{40} 16 hr before or at the same time as adenovirus produced 200 to 1,000 times more infectious virus than cells infected only with adenovirus, (ii) adenovirus multiplied faster in cells pre-infected with SV_{40} than in cells infected with both viruses simultaneously, and (iii) adenovirus multiplied at the increased rate regardless of whether SV_{40} DNA synthesis oc-

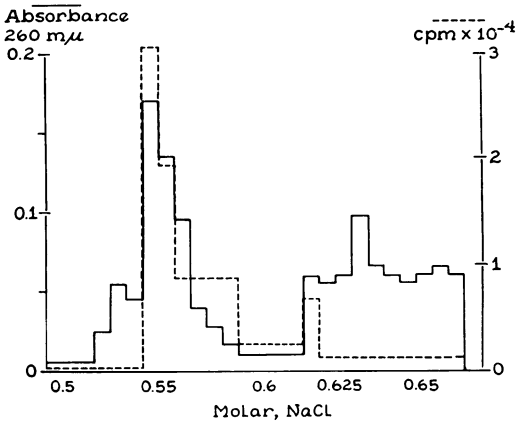

FIG. 7. *Chromatographic separation of DNA extracted from AGMK cells infected with type 2 adenovirus (3 PFU/cell) and SV_{40} (30 PFU/cell). Procedures for infection, isotopic labeling, extraction of DNA, and MAK chromatography were the same as described in Fig. 5 and 6.*

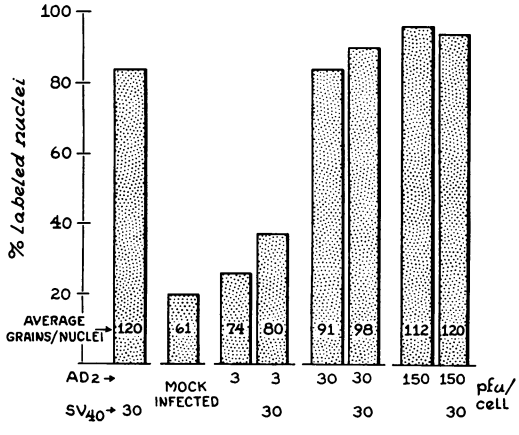

FIG. 8. *Proportion of cells labeled with ^{3}H-thymidine during infection with adenovirus type 2, SV_{40}, or combinations of both viruses. Confluent monolayers of AGMK cells on cover slips were infected; 18 hr after infection, 0.2 μc of ^{3}H-thymidine per ml was added, and the cultures were incubated for an additional 12 hr. Autoradiography was carried out on the cover slips. At least 1,000 cells per preparation were counted.*

curred during the 16 hr before infection with adenovirus.

The data summarized in Fig. 9 strengthen the hypothesis that infection of AGMK cells with SV_{40} induces the synthesis of a protein which is necessary for propagation of adenovirus, that the protein is not constitutive in the cells, and that its production cannot be brought about by adenovirus. To test this hypothesis directly, cycloheximide (50 μg/ml) was used instead of

TABLE 5. *Effect of infectious adenovirus, inactivated adenovirus, or soluble antigens on multiplication of SV40*

Group	Inoculum		Quantity of SV40[a] produced
	Adenovirus	SV40	
	PFU/cell	*PFU/cell*	*PFU/ml*
I	300	30	4.5×10^4
	150	30	1.5×10^5
	30	30	2.5×10^6
	3	30	9.3×10^6
	0	30	2.4×10^7
	Inactivated virus[b]	30	2.4×10^7
	Soluble antigens[c]	30	2.5×10^7
II[d]	150	30	3.5×10^5
	3	30	4.5×10^6
	0	30	2.3×10^7

[a] Infectivity was determined at 48 hr (group I) or 72 hr (group II) after infection.

[b] Hydroxylamine-inactivated adenovirus. Virus was incubated in 1 M hydroxylamine (*p*H 7.0 to 7.2) at 37 C for 24 hr.

[c] Partially purified mixture of hexon, fiber, and penton. The quantity employed was comparable, as measured in complement-fixation units, to the total amount of soluble antigen inoculated with 300 PFU/cell of crude virus.

[d] Adenovirus in this group was purified.

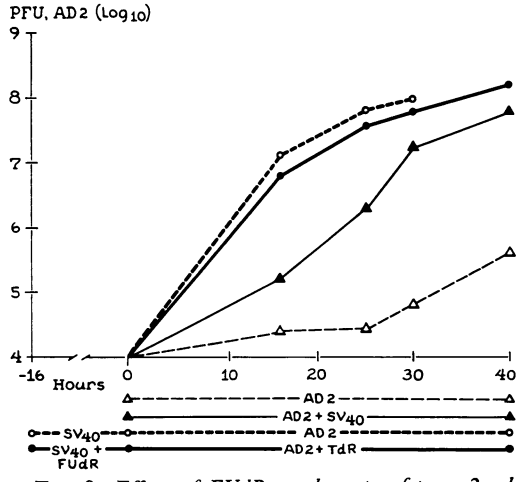

FIG. 9. *Effect of FUdR on the rate of type 2 adenovirus multiplication in cells preinfected with SV_{40}. Two sets of confluent monolayer cultures of AGMK cells (three cultures per set) were infected with SV_{40} at 30 PFU/cell. After infection, one set received 5×10^{-6} M FUdR. Sixteen hours after infection, both sets of cultures were infected with adenovirus (30 PFU/cell) and 5×10^{-6} M thymidine was added to the cultures which received FUdR. Two additional sets of cultures were infected, one with adenovirus and the other with adenovirus and SV_{40}. Replicate cultures of all four sets were assayed for infectious adenovirus.*

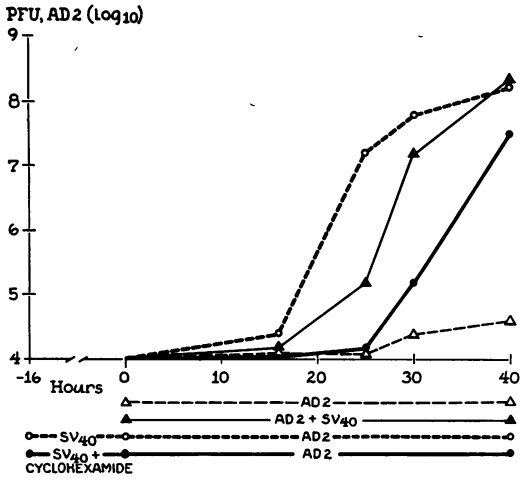

FIG. 10. *Effect of cycloheximide on the rate of type 2 adenovirus multiplication in cells preinfected with SV_{40}. Two sets of confluent monolayer cultures of AGMK cells were infected with SV_{40} (30 PFU/cells); 50 μg of cycloheximide per ml was added to one set of cultures. Sixteen hours after infection, cycloheximide was removed and both groups of cultures were infected with type 2 adenovirus, 30 PFU/cell. Two other groups of cultures were also infected at the same time with type 2 adenovirus and adenovirus plus SV_{40}. Three replicate cultures from each of the four sets were assayed for infectious type 2 adenovirus.*

5-FUdR to inhibit protein synthesis. Sixteen hours after SV_{40} infection and the addition of cycloheximide, the cultures were washed to remove the inhibitor from the affected cells and adenovirus was added. The results obtained (Fig. 10) demonstrate that, in the absence of protein synthesis, preinfection with SV_{40} did not increase the rate of adenovirus multiplication. The somewhat retarded appearance of adenovirus in cultures which had received cycloheximide reflects the delay in reversing the effect of the chemical on protein synthesis.

DISCUSSION

AGMK cells are restrictive hosts for adenovirus multiplication (28, 32) although a relatively small number of virions can be formed. The limiting event is not concerned with the initial events in infection of the cell since, except with low viral multiplicities, almost all cells can synthesize virus-specific early proteins. Nevertheless, the quantity of early proteins, and possibly even the number of early proteins, is increased when cells are concomitantly infected with SV_{40} and adenoviruses. Clearly the synthesis of capsid proteins is the major restriction in abortive adenovirus infection of AGMK cells. Even when co-infected with SV_{40}, approximately one-half the cells do not become permissive for complete adenovirus multiplication, but practically all cells synthesize DNA, the number of virions made is increased approximately 1,000-fold, and the production of capsid proteins is greatly augmented.

Because biosynthesis of adenovirus capsid proteins is dependent upon prior replication of viral DNA (12), it seemed possible that quantitatively viral DNA was limited in the singly infected cells. It had been reported that adenovirus DNA was synthesized even in the abortive infection (33), and Reich et al., with hybridization techniques, showed that in cells infected only with adenovirus approximately one-half the amount of adenovirus DNA was replicated as in cells infected with both viruses (35). Utilizing ultracentrifugation and chromatographic procedures, which permitted identification and quantitative recovery of the three species of DNA concerned, it was possible to demonstrate that approximately the same amount of adenovirus DNA was synthesized in the restricted as in the permissive infection, and that the quantity of viral DNA made was even far in excess of that essential for the number of virions assembled in the enhanced infection.

Investigation of the biosynthesis of DNA in singly and doubly infected cells revealed, however, that the viral interactions were more complicated than anticipated. As compared to a cell infected with SV_{40} alone, the kinetics of synthesis and the specific activity of the DNA were greatly altered in cells infected simultaneously with SV_{40} and adenovirus. Quantitative analyses of the species of DNA replicated in the singly and doubly infected cells revealed the basis for these striking alterations in cells infected with both viruses. (i) Synthesis of host DNA was not stimulated as in cells infected only with SV_{40}. (ii) Replication of SV_{40} DNA was inhibited, the degree of inhibition being dependent upon the multiplicity of adenovirus employed. (iii) Biosynthesis of adenovirus DNA was proportionate to the multiplicity of adenovirus infection and was similar to the amount synthesized in cells infected only with adenovirus.

The molecular basis is unknown for the biochemical conflict which blocks biosynthesis of both host and SV_{40} DNA while adenovirus DNA is replicated. It seems likely that the synthesis of host DNA is inhibited by a mechanism similar to the switch-off of host DNA synthesis in KB cells productively infected with type 5 adenovirus (15). Although the physical structure of SV_{40} DNA is strikingly different from that of mammalian cell DNA, the average base composition does not vary greatly; it is possible that the replication of both species of DNA is inhibited by

the same mechanism which is specified by the overall base structure or by certain similar nucleotide sequences. Alternatively, it is possible that initiation of SV_{40} DNA is a prerequisite for the enhanced synthesis of host-cell DNA. Hence, if the biosynthesis of SV_{40} DNA is inhibited, the increased synthesis of host DNA is not induced. However, in cells infected with adenovirus alone or with adenovirus and SV_{40}, there is significantly less biosynthesis of host-cell DNA than occurs in uninfected cells. It therefore seems likely that an active process blocks replication of host DNA rather than that its synthesis is merely not stimulated because synthesis of SV_{40} DNA is not permitted.

During the period before replication of SV_{40} DNA, the synthesis of one or more proteins is required for the enhanced production of adenovirus late proteins and thus the assembly of virions. Owing to its limited amount of genetic information, one may hypothesize that the protein is not a gene product of SV_{40} but rather that SV_{40} derepresses a host-cell protein which serves the required late adenovirus function. The hypothetical protein must be normally constitutive in KB cells and either constitutive in a small per cent of AGMK cells or present at a low level in many AGMK cells, thus permitting a small number of infectious adenovirus particles to be made under conditions of single infection. Alternatively, the genetic information necessary to synthesize the essential protein is present in the adenovirus genome, it is expressed in KB cells, but the function is restricted or the protein is inactivated in AGMK cells; infection with SV_{40} induces synthesis of a protein which may relieve the restriction or block the inactivation. This latter possibility could account for the finding that, with adenovirus alone, an increasing multiplicity of infection results in an increased yield of adenovirus. Thus, the presence of a greater number of functioning adenovirus genomes results in a greater opportunity for the postulated protein to participate in viral replication.

The intracellular interaction of SV_{40} and adenoviruses is not the sole example of biological and biochemical cooperation and conflict between animal viruses. For example, AAV are defective in all cells tested and their complete multiplication requires concomitant infection with an adenovirus (21, 29). However, while serving this helper function, the propagation of adenovirus is hindered (8, 30). The biochemical events responsible for the assistance and antagonism noted between viruses should elucidate some of the mechanisms which control the sequential transcription and translation of the genome in viral multiplication.

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