Replication in Simian Cells of Defective Viruses in an SV40-Adenovirus "Hybrid" Population

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Received for publication 18 September 1965

ABSTRACT

BUTEL, JANET S. (Baylor University College of Medicine, Houston, Tex.), AND FRED RAPP. Replication in simian cells of defective viruses in an SV40-adenovirus "hybrid" population. *J. Bacteriol.* 91:278-284. 1966.—An SV40-adenovirus type 7 "hybrid" virus population, previously shown to contain two viruses capable of complementation in green monkey kidney (GMK) cells, has a growth cycle in GMK cells similar to that of adenovirus type 7 in the presence of SV40. Extending previous preliminary results, the addition of adenovirus types 2, 7, or 12 to monolayers of GMK cells enhanced plaque formation by the SV40-adenovirus hybrid by as much as 200-fold. The terminal enhanced plaques, initiated by the hybrid in the presence of helper adenovirus, were found to contain progeny which could induce the synthesis of SV40 tumor antigen but which were coated with the protein of the helper adenovirus, type 2, 7, or 12, respectively. The particle carrying the SV40 tumor antigen determinant, named PARA, is defective in that it cannot direct the synthesis of capsid protein; information for the coat for PARA is supplied by the adenovirus. One-step growth curves of the hybrid virus population in monkey cells revealed that synthesis of both types of particles, adenovirus and PARA, proceeds at a similar rate, with a latent period of 16 to 20 hr being followed by an exponential increase in titer during the following 20 hr. Maximal titers for both particles were obtained 48 hr after inoculation of the cultures. Neither the PARA nor the adenovirus component replicated in GMK cells in the absence of the other.

An SV40-adenovirus type 7 "hybrid" population with several distinct properties has recently been described. ["Hybrid" is being used to denote a stable virus population possessing determinants of two distinct parental types but coated by the protein of one parent only. It does not imply a direct interaction between the nucleic acids of the parental types.] The virus can induce the synthesis of SV40 tumor (T) antigen in green monkey kidney (GMK) cells, but neither SV40 virus antigen nor infectious SV40 can be detected (10, 23, 26). The SV40 T antigen induced by the hybrid population is immunologically indistinguishable from that present during the early stages of the SV40 cytolytic cycle (9, 19, 22, 29) or from the T antigen synthesized in cells transformed by SV40 (3, 7, 17, 20). Induction of the SV40 T antigen and replication of the hybrid virus are both inhibited by adenovirus type 7 antiserum, but these properties are unaffected

by serum prepared against either SV40 or against the T antigen (10, 23, 26). Recent studies have demonstrated that the hybrid population consists of at least two distinct particles. Both are required to initiate plaque formation in GMK cells (27; Boeyé, Melnick, and Rapp, *Virology*, *in press*), and progeny derived from such plaques carry the SV40 determinant for T antigen (4, 21, 27). The determinant is not present, however, in plaque progeny obtained from human embryonic kidney (HEK) cells (4, 27). One of the particles appears to contain the genome of the adenovirus; the SV40 determinant is carried in the second particle (21; Boeyé et al., *in press*). Both particles, however, morphologically resemble adenovirions. The SV40 determinant can be transferred to other adenovirus types (21, 25) and it appears likely that the adenovirus supplies the information for the coat protein for both particles (21).

A study was therefore undertaken to delineate quantitatively the interaction between these particles during the infectious cycle of the hybrid virus in GMK cells. This report presents further evidence that SV40-adenovirus hybrid populations consist of two defective viruses which are mutually dependent on one another for growth in simian cells.

MATERIALS AND METHODS

Cell cultures. HEK cells were grown in lactalbumin hydrolysate (M-H) medium (13) supplemented with 10% fetal bovine serum (FBS). Maintenance medium for HEK cells consisted of M-H and 2% FBS. Primary kidney cells from African green monkeys (*Cercopithecus aethiops*) were grown in M-H containing 2% calf serum and were maintained in M-E (13) without serum.

Viruses. The SV40-adenovirus type 7 hybrid virus (stock SP2) has been described in detail (10, 23, 26). Two additional passages of the virus were carried out in GMK cells prior to this study. SV40 was the Baylor reference strain described in previous reports from this laboratory (19, 22). It was used after six passages in GMK cells.

Adenovirus type 7 (he) was isolated from the SP2 stock and plaque-purified three times in HEK cells by Boeyé et al. (4). It was used after four additional passages in HEK cells and is free from SV40 T antigen determinants. Another strain of adenovirus type 7 (hu) was isolated from a fatal case of pneumonia (1) and has been passed only in HEK cells; it, too, does not contain detectable SV40 determinants.

Adenovirus type 2 was also a fresh human isolate and was supplied by M. Benyesh-Melnick. It was used after three passages in KB cells. Adenovirus type 12 was obtained originally from R. J. Huebner. It has been passed numerous times in KB cells and once in HEK cells. Neither virus carries detectable SV40 determinants.

All virus stocks were prepared in cells growing in 16-oz (454-g) bottles; virus was harvested by disrupting the cells in the medium. Cell debris was removed by low-speed centrifugation, and the supernatant fluid was dispensed in 1-ml amounts into glass ampoules; the ampoules were sealed, and the virus was then quick-frozen and stored at -90°C .

Antisera. Neutralizing antisera were prepared in rabbits against the type 7 (hu), type 2, and type 12 adenoviruses described above. Rabbits received one intramuscular injection weekly for 3 weeks, a fourth inoculation 2 weeks later, and were bled 2 weeks after the final inoculation.

Virus assays. Adenoviruses were titrated in HEK cells grown as monolayers in 35-mm plastic petri dishes (4, 6). The cultures were incubated at 37°C in a 5% CO_2 incubator. The hybrid virus was also assayed on GMK cell monolayers grown in 60-mm plastic petri dishes (4, 21). In both assays, 0.1 ml of virus inoculum was allowed to adsorb for 1 hr at 37°C with frequent manual rotation; the 60-mm plates also re-

ceived 0.2 ml of tris(hydroxymethyl)aminomethane (Tris) buffer as carrier fluid to facilitate even distribution of the virus. The overlay consisted of Eagle's basal medium, 10% FBS, 1% agar, and 0.23% sodium bicarbonate; 7 days later, a second overlay containing a 1:20,000 dilution of neutral red was added. Plaques were visible the next day, but maximal titers were not reached until the 11th to the 13th day after inoculation of the cultures. All titrations were carried out with two or three plates per dilution.

Enhancement experiments involving GMK cells were performed in the same manner as the assay, except that 0.1 ml of a high concentration of the helper adenovirus was inoculated and allowed to adsorb simultaneously with the inoculum being titrated.

Immunofluorescence techniques. Secondary cultures of GMK cells were grown on 15-mm cover slips in plastic petri dishes; 24 hr after being inoculated, the cells on the cover slips were washed with warm Tris buffer (pH 7.4) and fixed for 3 min in acetone. SV40 T antigen was detected (22) by treating the cells first with sera from hamsters bearing SV40-induced tumors and, second, with anti-hamster globulin prepared in rabbits and labeled with fluorescein isothiocyanate. Detailed fractionation and labeling methods have been described (12). A Zeiss fluorescence microscope, with an Osram HBO-200 mercury arc vapor lamp for illumination, was used to examine the stained preparations. Each test included appropriate positive and negative controls.

RESULTS

Replication of an SV40-adenovirus type 7 hybrid in GMK cells. Replicate GMK cultures were infected with the hybrid (SP2) virus at an input multiplicity of 3 plaque-forming units (PFU)/cell, based on the SP2 titer obtained in HEK cells. Adenovirus type 7 (he) was inoculated onto GMK monolayers at an input multiplicity of 4 PFU/cell. Replicate cultures inoculated with adenovirus type 7 (he) also received SV40 at a multiplicity of infection of 2 PFU/cell. Virus was harvested from the three series of infected cultures at various times after inoculation, and adenovirus titers were obtained by plaquing on HEK cells. Adenovirus type 7 (in cultures inoculated only with that virus) gradually decreased in titer during the 72-hr observation period (Fig. 1); the rate of decrease paralleled the rate of thermal inactivation for adenovirus 7 at 37°C . However, adenovirus type 7, in the presence of SV40, replicated after a latent period of about 20 to 24 hr. Virus yields were nearly maximal 48 hr after inoculation of the cultures. The SP2 population replicated in a pattern closely parallel to that of the nonhybridized adenovirus 7 in the presence of SV40. Again, the latent period was about 20 to 24 hr, and maximal

FIG. 1. Replication in green monkey kidney cells of an SV40-adenovirus 7 hybrid virus (SP2) and of adenovirus 7 (in the presence and absence of SV40).

virus yields were obtained 48 hr after inoculation of the cultures.

Enhancement by adenoviruses of SP2 plaque titers in GMK cells. Monolayers of GMK cells were exposed to various types of adenoviruses and were inoculated simultaneously with dilutions of SP2. The concentration of adenovirus used was the highest dilution of stock virus giving maximal enhancement in the absence of cytotoxicity. The adenoviruses alone did not produce any lesions in the cell sheets, but the SP2 plaque titers were enhanced 30- to 200-fold by the addition of the noncytopathic (in GMK cells) adenoviruses (Table 1). Enhancement could be provided by types 2, 7, and 12. Thus, there was not a specific requirement for the homologous type 7.

Heat-inactivated adenoviruses did not enhance plaque formation by SP2. SV40 also failed to enhance plaque titers of SP2. Filtration through Gradocol membranes revealed that the enhancing particle is in the size range of an adenovirus. The particle which interacted with the adenovirus to initiate plaque formation has previously been

named PARA (Particle Aiding, and aided by, the Replication of Adenovirus; 21).

Transfer of PARA from adenovirus type 7 (SP2) to other adenoviruses. Under conditions of maximal enhancement with various adenoviruses, plaques were usually initiated by SP2 at dilutions 100-fold beyond the end point obtained in the absence of helper adenovirus. These terminal plaques were picked, the particles were passaged in GMK cells, and the progeny were studied. This was done to determine the nature of the particles which had interacted to form the plaques. All 35 plaques analyzed yielded progeny which were capable of inducing in GMK cells the synthesis of SV40 T antigen, detectable by immunofluorescence procedures. Neutralization tests revealed that the induction of the T antigen was not necessarily inhibited by antiserum against adenovirus 7, but rather by antiserum directed against the specific adenovirus which had acted as the enhancing virus (Table 2). Progeny from SP2 plaques formed by enhancement with adenovirus type 2 were no longer antigenically type 7 adenoviruses, but were neutralized as though they were bearing the protein coat characteristic of adenovirus type 2. The progeny from one adenovirus type 2-enhanced plaque induced T antigen synthesis after neutralization with either adenovirus type 2 or type 7 antiserum. That population probably consisted of a mixture of antigenic types. Similar results were obtained with the progeny from adenovirus type 12-enhanced plaques, with the exception that three plaques yielded progeny which were still coated with adenovirus type 7 protein. These particular plaques had been picked at higher concentrations of hybrid inoculum because the concentration of co-infecting adenovirus 12 had not provided maximal enhancement. This technical problem undoubtedly increased the chances of picking plaques which had been initiated by superinfection with adenovirus type 7 from the SP2 inoculum.

TABLE 1. Enhancement by adenoviruses of plaque formation by an adenovirus-SV40 hybrid (SP2) in green monkey kidney cells

Enhancing virus	SP2 titer ($\log_{10}$ PFU/ml)	
	Expt 1	Expt 2
None.....	4.93	4.85
Adenovirus type 7.....	6.60	6.34
Adenovirus type 2.....	7.23	6.90
Adenovirus type 12....	Not done	6.46

TABLE 2. *Neutralization (of ability to induce synthesis of SV40 T antigen in GMK cells) of progeny from adenovirus-enhanced SP2 plaques*

Enhancing virus	SP2 plaque progeny neutralized with*			
	Tris	Anti-adeno 7	Anti adeno 2	Anti-adeno 12
Adenovirus type 7	9/9	0/9	Not done	Not done
Adenovirus type 2	12/12	12/12	1/12	Not done
Adenovirus type 12	14/14	11/14	Not done	3/14

* Numerator = number of plaques whose progeny induced SV40 T antigen detectable by immunofluorescence after neutralization; denominator = number of plaques tested; adeno = adenovirus.

TABLE 3. *Neutralization (of ability to induce plaque formation in GMK cells) of progeny from adenovirus-enhanced SP2 plaques*

Neutralized with	Avg no. of plaques per plate			
	Parent SP2		Adenovirus 2-PARA*	Adenovirus 12-PARA†
	Expt 1	Expt 2		
Tris buffer.....	94	20	30	94
Adenovirus 7 antiserum.....	0	0	23	86
Adenovirus 2 antiserum.....	Not done	15	0	86
Adenovirus 12 antiserum.....	100	Not done	38	0

* Adenovirus 2-PARA = progeny from terminal SP2 plaque formed by enhancement with adenovirus type 2.

† Adenovirus 12-PARA = progeny from terminal SP2 plaque formed by enhancement with adenovirus type 12.

The ability of the progeny to plaque in GMK cells was also neutralized by appropriate antiserum. The results obtained are also in agreement with the hypothesis that the progeny from enhanced SP2 plaques acquire the antigenic properties of the helper virus. Numerous plaque progeny were tested; representative data are shown in Table 3. Antiserum prepared against the human strain of adenovirus type 7 (hu) inhibited plaque formation by the parent SP2, but was without effect on progeny from terminal SP2 plaques formed by enhancement with heterologous adenoviruses. The latter progeny were, however, neutralized by specific antiserum against the enhancing virus, either adenovirus type 2 or type 12 (Table 3).

Neutralization of PARA by specific antiserum. Since PARA could not plaque on GMK cells in the absence of co-infecting adenovirus, the possibility existed that the neutralization results shown in Table 3 were due merely to the neutralization of the helper virus. It was necessary to show that PARA itself was neutralized, in order to support the conclusion that it was encased in an adenovirus capsid (21, 25; Boeyé et al., *in press*). This was accomplished by neutralizing the parent SP2 stock with adenovirus 7 antiserum,

and then plating the neutralized mixture on GMK cells simultaneously inoculated with adenovirus type 2. Thus, if PARA were in a distinct type of protein coat, different from that of adenovirus 7, plaques should develop in the presence of the provided helper virus. This did not occur, although preparations of SP2 not treated with anti-adenovirus 7 serum showed the typical enhancement phenomenon in the presence of helper adenovirus type 2 (Table 4). Rabbit antiserum prepared against adenovirus type 12 served as a control, and had no neutralizing effect. These results indicate that PARA in the SP2 population is encased in an adenovirus type 7 protein coat. Analogous results have been obtained with PARA-adenovirus type 2 and PARA-adenovirus type 12 populations.

Mutual dependence between PARA and adenovirus for replication in GMK cells. The above results indicated that plaques formed in GMK cells after the inoculation of SP2 (or PARA in the presence of helper adenoviruses) always contained both PARA and adenovirus particles. A study was undertaken to determine quantitatively the replication of PARA and adenovirus.

Replication of adenovirus. Adenovirus 7 alone

TABLE 4. Neutralization of PARA in the SP2 population with adenovirus type 7 antiserum

Inoculum	Avg no. of plaques per GMK plate	
	No helper virus present	Helper adenovirus* present
SP2 + Tris buffer.....	3	>100
SP2 + adenovirus 12 antiserum..	3	>100
SP2 + adenovirus 7 antiserum...	0	1

* Adenovirus type 2.

does not multiply in GMK cells (6; Feldman, Butel, and Rapp, *J. Bacteriol.*, *in press*). The adenovirus in the SP2 population does multiply in GMK cells (Fig. 1) in the presence of the defective SV40 component (PARA).

Replication of PARA. We have been unable to physically separate PARA and adenovirus in the hybrid virus population. This rendered studies of PARA replication more difficult. Several experimental approaches were therefore used.

Approach 1: GMK cells in 1-oz (28-g) bottles (about 10^6 cells) were infected with SP2 at a dilution calculated to introduce about 100 PFU of PARA per culture. The inoculum at this dilution contained about 50 PFU of adenovirus 7. This low input multiplicity should have resulted in some cells being singly infected with PARA. Replicate bottles were inoculated with 4 PFU/cell of adenovirus type 7 (he) and 100 PFU of PARA per culture. This was to ensure that every PARA-infected cell would be jointly infected with adenovirus. Harvests were made 7 and 96 hr after infection, and were titrated in GMK cells saturated with helper adenovirus to titrate for PARA. Undiluted harvests taken 7 hr after inoculation did not cause the formation of plaques. Likewise, the undiluted 96-hr harvest from the culture inoculated only with SP2 did not yield plaques. However, the 96-hr harvest, from the culture receiving *both* SP2 and additional adenovirus type 7 contained 800 PFU of PARA per culture. Similar experiments, with adenovirus type 2 as helper virus, gave comparable results. Therefore, PARA replication in the presence of adenovirus was detected, but none could be detected in the absence of joint infection with adenovirus under otherwise identical conditions.

Approach 2: The harvests from the SP2 growth in GMK cells used to study adenovirus replication (Fig. 1) were titrated in GMK cells co-infected with helper adenovirus to enumerate PARA particles. The initial inoculum for this experiment

contained about 2 PFU of PARA per cell and 4 PFU of adenovirus 7 per cell. Under conditions of joint infection, PARA underwent a cycle of replication (Fig. 2) closely paralleling that of the helper adenovirus 7 in the same system (sketched in from Fig. 1 for comparison purposes).

Approach 3: GMK monolayers were inoculated with low multiplicities of SP2 and the cultures were incubated under fluid medium. Saturating amounts of helper adenovirus were added on successive days after infection, at which time an agar overlay was also added. If PARA could replicate unilaterally, spread and reinfection throughout the cultures should have occurred and those cultures maintained under fluid for progressively longer periods of time prior to the addition of adenovirus should have yielded increasing plaque counts. PARA titers obtained 13 days after the adenovirus was added are shown in Table 5. It can be seen that PARA persisted at a near-constant level for 24 hr in the GMK cells, but the addition of adenovirus at later intervals revealed a progressive drop in titer. This suggests that PARA had not replicated until the adenovirus was added and had been gradually inactivated in the GMK cells prior to the addition of the adenovirus.

These experimental approaches, therefore, yielded evidence compatible with the concept that

FIG. 2. Replication of adenovirus 7 and PARA in green monkey kidney cells inoculated with an SV40-adenovirus 7 hybrid.

TABLE 5. Absence of PARA replication in green monkey kidney cells prior to the addition of adenovirus type 7 (he)

Adenovirus added (hours after infection)	Avg no. of PARA plaques per GMK plate*
0	51
24	47
48	21
72	6

* Readings taken 13 days after inoculation of the adenovirus; average of two plates per group.

neither adenovirus type 7 nor PARA can replicate on its own in GMK cells, although each does so in the presence of the other.

DISCUSSION

Heterologous viruses are capable of various types of interaction when both are replicating within the confines of a single cell. When one intact genome becomes encased within a protein capsid coded for by the other virus, the phenomenon is referred to as phenotypic mixing. As there is no genetic defect or alteration, the phenotypically mixed particles do not breed true but induce the proper protein coat on subsequent passage. The SV40-adenovirus hybrids are not examples of phenotypic mixing, because complete SV40 has not appeared after numerous subcultures of the stock virus.

Recombination occurs when a portion of the genome of one virus becomes attached to the genetic material of a second. The progeny derived from such a virus will possess properties of each parent, and the recombinants breed true on passage. Initially, it had been suggested that the SP2 population might have stemmed from a recombination of the genomes of SV40 and adenovirus type 7. In view of the demonstration here and elsewhere (21, 25) that the adenovirus 7 portion of the population can easily be replaced by another type of adenovirus, it appears unlikely that recombination has occurred. The possibility that an undetectable portion of the adenovirus genome might actually be attached to the defective SV40 DNA cannot yet be ruled out, however.

Complementation may be defined as the functional interaction between two viruses, such that replication occurs under otherwise inhibitory conditions. As long as the two viruses are not defective in the same coding region, complementation can occur. It has been studied most extensively with bacteriophages (2, 14, 30, 31).

A virus which requires complementation in

order to replicate is defective in some way. An example is λ dg bacteriophage. It has replaced a portion of its genome with part of the *gal* genetic region of *Escherichia coli* (5). Satellite tobacco necrosis virus (STNV) is smaller and serologically unrelated to tobacco necrosis virus, but replicates only in its presence (11). Reichmann (24) found that STNV contains only enough ribonucleic acid to code for 400 amino acids and that the coat protein subunit consists of 372 amino acids. He suggested that one protein is all the satellite virus can code for and that the satellite virus is dependent upon replicating necrosis virus for other needed factors.

The Bryan strain of Rous sarcoma virus (RSV) is a defective animal virus. It cannot direct the synthesis of coat protein and is dependent upon a helper virus, RAV, for its production (8). Similarly, adenovirus must supply the capsid for PARA. The two systems are not strictly analogous, however, because both RSV and RAV can exercise independent functions in the absence of the other (28). RSV can transform cells, and RAV can undergo a complete replicative cycle. We have been unable to detect any such perpetuating activity in simian cells by PARA or adenovirus, and neither can replicate in this type of cell independently. The defect which prevents adenovirus replication in monkey cells has not yet been determined. During joint infection, SV40 potentiates adenovirus multiplication (6, 15, 16, 18; Feldman et al., *in press*). PARA serves the same function during replication of the adenovirus-SV40 hybrid populations.

The conversion of the capsid protein of PARA from that of one adenovirus type to that of another, with the retention of the SV40 determinants in a stable form, has been called trans-capsidation (21).

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grants CA-04600 from the National Cancer Institute and AI-05382, 5 TI AI 74, and AI-05398 from the National Institute of Allergy and Infectious Diseases.

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