Characterization of the Strain of Adenovirus Type 7 Carrying the Defective Monkey Cell-adapting Component

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The strain of adenovirus type 7 carrying the defective monkey cell-adapting component (MAC) has been further characterized. MAC is more sensitive to inactivation by ultraviolet light than the associated adenovirus, which, in turn, is more rapidly inactivated than complete simian virus 40 (SV40). The 37% dose was 16 sec for MAC, 60 sec for adenovirus, and 84 sec for SV40. Filtration through membranes revealed that both MAC and adenovirus were retained by 100-m μ filters. MAC equilibrated in cesium chloride at the same buoyant density as the complete adenovirions (1.34 g/ml). Deoxyribonucleic acid extracted from green monkey kidney cells infected with MAC-adenovirus 7 did not hybridize in vitro with SV40 complementary ribonucleic acid. The two components (MAC and adenovirus) of the virus population possess such similar biophysical properties that their separation has not yet been achieved. Evidence to date indicates that MAC is probably not derived from SV40.

Human adenoviruses are not able to replicate in cells of simian origin unless the cultures are co-infected with simian papovavirus SV40. The necessary growth-potentiating information can be supplied by either defective (5, 12, 26, 30) or complete (1, 9, 15) SV40 genomes encased in adenovirus capsids, as well as by autonomously replicating SV40 (10, 16, 20, 21). Recent surveys of strains of human adenoviruses adapted to growth in monkey kidney cells revealed that they were contaminated with SV40, as detected by the induction of SV40 tumor (T) antigen (14, 18).

A unique strain of adenovirus type 7 has recently been reported (8). This particular virus is adapted to growth in monkey kidney cells, but does not appear to carry any SV40 determinants. It does not induce the synthesis of SV40 tumor or virus capsid antigens (8) and does not confer SV40-specific transplantation immunity to weanling hamsters (24).

However, the adapted adenovirus 7 population contains a defective particle which can be transferred to heterologous serotypes of human adenoviruses (7, 8) by a process called transcapsidation (23). The addition of the defective particle enables human adenoviruses to replicate in simian cells (7, 8). The particle was, therefore, named the monkey cell-adapting component or MAC (8).

MAC is of interest since it does not induce any recognizable SV40-specific antigens and is, therefore, different from any other defective genome known to be carried in adenovirus capsids. A physical separation of MAC from the helper adenovirions would greatly facilitate characterization of the defective particle. This report summarizes the results of a characterization of the two components of the adapted adenovirus 7 population initiated in an attempt to devise a means for such a physical separation. Biophysical evidence is included which further indicates that MAC is not derived from SV40.

MATERIALS AND METHODS

Viruses. The history of the parental adapted virus, originally designated adenovirus 7 (M), has been described in detail (8). This population carries the defective monkey cell-adapting component and, hence, will be referred to as MAC-adenovirus 7. The virus was used after two passages in our laboratory in green monkey kidney (GMK) cells. The virus stock was free of adeno-associated satellite virus particles (17) when viewed with an electron microscope.

Adenovirus 7 (PP3) routinely used as helper virus in the plaque assay for MAC in GMK cells was derived from the SV40 adenovirus "hybrid" population by plaque purification in human embryonic kidney (HEK) cells (2). It has been passed five additional times in HEK cells, does not induce the synthesis of

SV40 antigens, and does not replicate in GMK cells. The HEK cells were supplied by the Human Tissue Procurement Program, National Cancer Institute.

SV40 was the Baylor reference strain used in previous studies from this laboratory (22, 25). It has been passed seven times in GMK cells.

Virus stocks were prepared in cells growing in 16-oz (453-ml) bottles. When cytopathic effects (CPE) involved 75 to 100% of the cell monolayer, the cells were disrupted into the medium by two alternate cycles of quick-freezing in a dry ice-alcohol bath, followed by thawing. The suspension was treated in a Raytheon sonic oscillator for 30 sec and then clarified by low-speed centrifugation. The supernatant fluid was dispensed in 1-ml amounts into glass ampoules, which were then sealed. The contents were quick-frozen and stored at -90°C .

Preparation and purification of radioactive-labeled virus. A stock of MAC-adenovirus 7 labeled with tritiated thymidine (^3H -TdR) was prepared as follows. GMK cells in 16-oz bottles were prelabeled for 24 hr with serum-free Eagle's medium containing $3\text{ }\mu\text{C}$ of ^3H -TdR/ml ($30\text{ }\mu\text{C}$ of ^3H -TdR/bottle). After adsorption of the virus, 12 ml of serum-free Eagle's medium containing $10\text{ }\mu\text{C}$ of ^3H -TdR/ml was added to each bottle. When virus CPE was extensive, the radioactive medium was removed and 10 ml of unlabeled medium was added to each bottle. The cells were disrupted by quick-freezing and thawing, and the suspension was then treated in a sonic oscillator for 60 sec.

Virus was sedimented from 25 ml of crude virus suspension by centrifugation for 3 hr at 30,000 rev/min with the use of a 30 rotor in a model L ultracentrifuge. The virus pellet was allowed to dissolve overnight at 4°C into 1 ml of 0.01 M tris(hydroxymethyl)aminomethane (Tris) buffer, pH 8.1. This suspension was then dialyzed for 6 hr at 4°C against 0.01 M Tris buffer, pH 8.1, after which it was treated for 45 min at 37°C with $20\text{ }\mu\text{g}$ of deoxyribonuclease/ml in the presence of $3 \times 10^{-3}\text{ M}$ MgCl_2 .

The virus-enzyme mixture was then subjected to differential centrifugation with the use of a preformed gradient of cesium chloride. Centrifugation was for 18 hr at 39,000 rev/min with an SW50L rotor in a model L ultracentrifuge. The virus band was harvested by side puncture and diluted with Tris buffer to a density of 1.28 g/ml. This was layered immediately onto a new preformed gradient of cesium chloride and centrifuged for 22 hr at 39,000 rev/min in a model L ultracentrifuge with an SW50 L rotor. Ten-drop fractions were collected from the bottom of the tube, the refractive indices of these fractions were determined and converted to buoyant density, and the radioactivity was assayed by counting 0.02 ml portions of the fractions in toluene scintillation fluid (4) by use of a liquid scintillation spectrometer (CPM 200, Beckman Instruments).

Virus assays. Infectious adenovirus was assayed by the plaque technique in HEK cells as described (2, 5). The agar overlay consisted of Eagle's medium, 10% fetal bovine serum, and 0.23% sodium bicarbonate. A second overlay, which also contained a 1:20,000 dilution of neutral red, was applied on the 8th day.

SV40 was assayed in GMK cells in 60-mm plastic petri dishes as reported previously (6). The same Eagle's overlay as described above for the adenovirus assays was used.

The assay for MAC in GMK cells has also been described (7). Adenovirus 7 (PP3) was routinely employed as the helper virus. The Eagle's overlay described above was used.

Ultraviolet (UV) light inactivation. The same procedure was used as previously described (6). The technique was modified so that the bulb (Westinghouse Sterilamp 615T3) was raised to a height of 32 cm above the samples, and the viruses were diluted 1:2 with Tris buffer prior to irradiation.

Nucleic acid hybridization. These experiments were performed in collaboration with Sherman M. Weissman. The techniques used to detect hybridization of deoxyribonucleic acid (DNA) with complementary

FIG. 1. Suspensions of MAC-adenovirus 7 and of SV40 were exposed to ultraviolet light for various lengths of time. The irradiated samples were then tested for surviving infectivity by the plaque technique. SV40 was assayed in green monkey kidney (GMK) cells, adenovirus in human embryonic kidney cells, and MAC in GMK cells co-infected with helper human adenovirus 7 (PP3).

ribonucleic acid (RNA) have been described (28). Radioactive complementary RNA was synthesized *in vitro* with the enzyme, RNA polymerase, and DNA from purified virus or from uninfected GMK cells as primer. After incubating the DNA and complementary RNA under conditions favoring hybridization, the solutions were filtered and the filters were then treated for 60 min at 25 C with 5 ml of a solution containing pancreatic ribonuclease at a concentration of 20 $\mu\text{g}/\text{ml}$.

TABLE 1. *Size determination of MAC-adenovirus 7 by filtration^a*

Filter pore size (m μ)	PFU/ml in filtrate	
	Adenovirus 7	MAC-7
Unfiltered	2.1×10^4	2.2×10^3
220	2.5×10^4	2.3×10^3
100	$<1.0 \times 10^2$	$<1.0 \times 10^1$
50	$<1.0 \times 10^1$	$<1.0 \times 10^1$

^a MAC-adenovirus 7 stock was diluted 1:1,000 with distilled water, and samples were then passed through filters (Millipore Corp.) of varying porosities. The filtrates were titrated for adenovirus plaque-forming units (PFU) in human embryonic kidney cells and for MAC infectivity in green monkey kidney cells co-infected with helper human adenovirus type 7 (PP3).

RESULTS

Susceptibility to UV light. Susceptibility to inactivation by UV light was determined for both members of the MAC-adenovirus 7 population and for complete SV40. Virus suspensions were irradiated for various lengths of time, and the surviving infectivity was assayed in appropriate cell cultures. Results from typical experiments are shown in Fig. 1. After exposure to UV light for 1 min, only 1.6% of the initial infectivity of MAC-7 remained, as compared with 37% for the associated adenovirus 7 and a surviving fraction of 50% for complete SV40. The 37% dose (13), the dose of irradiation which leaves 37% of the virus population as a surviving fraction ($e^{-1} = 37\%$), was 16 sec for MAC-7, 60 sec for adenovirus 7, and 84 sec for SV40, under the conditions of these experiments.

These results are similar to those obtained previously with PARA-adenovirus 7 (6). The known defective SV40 particle (PARA) was found to be more rapidly inactivated by UV light than either the helper adenovirus 7 or complete, parental SV40. The inactivation rates for adenovirus and SV40 were more rapid in those experiments because the source of the UV light was closer to the virus samples.

Sensitivity to thermal inactivation at 50 C

FIG. 2. Stock of MAC-adenovirus 7 was centrifuged to equilibrium in a gradient of cesium chloride. Droplet fractions were collected and buoyant densities determined based on refractive index readings. The fractions were then plaqued in both human embryonic kidney cells to detect infectious adenovirus and in green monkey kidney cells co-infected with helper human adenovirus 7 (PP3) to assay MAC infectivity.

FIG. 3. A purified preparation of MAC-adenovirus 7 labeled with tritiated thymidine was centrifuged to equilibrium in a gradient of cesium chloride. Droplet fractions were collected, their refractive indices were taken and converted to buoyant densities, and the fractions were then analyzed for content of radioactivity.

TABLE 2. Ability of DNA from GMK cells infected with MAC-adenovirus 7 to hybridize with various complementary RNA preparations^a

DNA	Counts retained of RNA complementary to			Ratios	
	GMK	SV40	PARA-adenovirus 7	SV40/GMK	PARA-adenovirus 7/GMK
Expt 1					
GMK + MAC-adenovirus 7.....	2,763 ^b	65.5	2,840.0	0.023	1.021
GMK control.....	4,937	101.0	121.0	0.021	0.025
Expt 2					
GMK + MAC-adenovirus 7.....	6,603	114.0	—	0.017	—
GMK control.....	3,726	94.0	—	0.029	—
Mycoplasma.....	—	20.0	26.5	—	—
No DNA.....	15	17.2	16.0	—	—

^a DNA extracted from either uninoculated control green monkey kidney (GMK) cells or from GMK cells infected with MAC-adenovirus 7 was incubated with radioactive complementary RNA. There were 305,000 counts/min of GMK cell complementary RNA added per vial, 475,000 counts/min of SV40 complementary RNA, or 520,000 counts/min of PARA-adenovirus 7 (SV40-adenovirus "hybrid") complementary RNA per vial. The number of counts retained in each case, and the ratios of counts retained of virus complementary RNA compared to the retention of GMK complementary RNA, are shown above.

^b Each number is an average of two assays.

was also determined for the members of the MAC-adenovirus 7 population. The rates of inactivation for adenovirus and MAC were similar. This was to be expected, however, since both particles immunologically appear to be encased in identical protein coats (7, 8).

Size of virus by filtration. The MAC-adenovirus 7 stock was diluted 1:1,000 in sterile water to

prevent nonspecific adsorption of the virus to filters (32), and samples were passed through membrane filters (Millipore Corp., Bedford, Mass.) of varying porosities. The filtrate in each case was assayed for adenovirus and MAC by the plaque technique. It can be seen in Table 1 that both the adenovirus and the MAC in the population passed through a 220-m μ filter with

no loss of infectivity. However, both components were retained by 100- and 50-m μ filters. No infectivity was detected in these filtrates. These results support the concept that the capsid containing the MAC genome is not significantly larger or significantly smaller than an adenovirus capsid.

Buoyant density of the virus. An attempt was made to separate physically the two particles on the basis of buoyant density. The crude virus stock was layered onto a preformed gradient of cesium chloride and centrifuged to equilibrium (39,000 rev/min for 18 hr). Fractions were collected from the bottom of the tube, their refractive indices determined, and the buoyant densities were calculated. The fractions were then assayed for MAC and adenovirus plaque-forming abilities in appropriate cell cultures. The results are shown in Fig. 2. The infectious units of adenovirus and of MAC were distributed in a similar pattern throughout the gradient. The infectivity of each member of the population peaked in one fraction which had a buoyant density of 1.34 g/ml. Centrifugation to equilibrium did not alter the ratio of approximately 100 adenovirus plaque-forming units for each plaque-forming unit of MAC.

A density gradient centrifugation experiment was also performed with radioactive-labeled virus as described in Materials and Methods. Fractions from the gradient were analyzed for radioactivity. As shown in Fig. 3, the bulk of the radioactivity was found in a single fraction with a buoyant density of 1.34 g/ml.

The preceding results indicate that MAC and adenovirus cannot be physically separated on the basis of the buoyant densities of the particles.

Homology studies. Nucleic acid homology studies were carried out in an effort to determine whether MAC contained SV40-specific DNA even though it is defective for SV40 known antigens. DNA was extracted from uninoculated GMK cells and from GMK cells infected with MAC-adenovirus 7. Such cellular DNA preparations were tested against virus-complementary RNA and against GMK-complementary RNA for ability to hybridize. The results are expressed as the ratio of the counts per minute retained in the two assays (Table 2). The specificity of the reactions involving the complementary RNA preparations has been demonstrated (28).

DNA from both the control and infected GMK cells reacted with GMK complementary RNA, as expected. There was no increased reactivity with SV40 RNA when the DNA was extracted from cells infected with MAC-adenovirus 7. In both cases, the SV40-GMK ratio was low (about 0.02). In previous experiments (28), it

had been shown that DNA extracted from GMK cells doubly infected with SV40 and human adenovirus type 7 reacted as well with SV40 RNA as with GMK RNA, resulting in an SV40-GMK ratio of at least 1.0. Therefore, the cells infected with MAC-adenovirus 7 would appear to contain very little, if any, replicating SV40 DNA.

The DNA from GMK cells infected with MAC-adenovirus 7 did react with RNA complementary to PARA-adenovirus 7. This is presumably because adenovirus DNA was synthesized in the infected cultures. The PARA-7-GMK ratio was 1.02. Control cell DNA did not react with this RNA, giving a PARA-7 to GMK ratio of only 0.02. *Mycoplasma pneumoniae* DNA was used for control purposes and did not react with SV40 or PARA-7 complementary RNA.

DISCUSSION

This paper reports certain biological and biophysical characteristics of the strain of adenovirus type 7 containing the defective monkey cell-adapting component. The two particles (MAC and adenovirus) present in this population adapted to growth in monkey kidney cells have such similar properties that a physical separation of the two has not been achieved.

Evidence currently available indicates that MAC is probably not derived from SV40. However, MAC is similar in many respects to a known defective SV40 particle, called PARA. Since both these defective viruses are found associated with strains of human adenoviruses adapted to growth in simian cells, it is useful to compare certain properties of the two particles (Table 3).

As previously mentioned, both PARA (5, 11) and MAC (7, 8) enable human adenoviruses to replicate in GMK cells in the absence of SV40. These particles, in turn, are dependent upon the helper adenoviruses for replication (3, 5, 7, 31) because the particles are defective for capsid protein. PARA (12, 26, 30) and MAC (7, 8) are encased in capsids synthesized by the helper adenovirions. Because of their inability to synthesize coat protein, both PARA (23, 29) and MAC (7, 8) can be transcapsidated to other serological types of adenoviruses. PARA (2) and MAC (7) are removed by plaque purification of the adenovirus populations in human cells.

As previously reported for PARA (6), MAC has a buoyant density comparable to that of complete adenovirions (1.34 g/ml in CsCl) and is more sensitive to inactivation by UV light

TABLE 3. *Comparison of defective viruses PARA and MAC^a*

Property	PARA ^b	MAC ^c
Enhances human adenoviruses in GMK cells ^d	+	+
Dependent on helper adenovirus for replication	+	+
Encased in adenovirus capsids	+	+
Can be transcapsidated to other adenoviruses	+	+
Removed by plaque purification in HEK cells	+	+
Buoyant density = 1.34 g/ml in CsCl	+	+
More sensitive to UV than adenovirus or SV40	+	+
Induces synthesis of SV40 V antigen	0	0
Induces synthesis of SV40 T antigen	+	0
Confers SV40 transplantation immunity	+	0
DNA hybridizes in vitro with SV40-specific RNA	+	0

^a Part of this table is taken from reference 7.

^b PARA = defective SV40 carried by some adenovirus populations able to replicate in simian cells.

^c MAC = defective monkey cell-adapting component carried by strain of adenovirus 7 characterized in this report.

^d Abbreviations: GMK, green monkey kidney; HEK, human embryonic kidney; CsCl, cesium chloride; UV, ultraviolet; SV40 V, viral (capsid) antigen; SV40 T, tumor antigen; DNA, deoxyribonucleic acid; RNA, ribonucleic acid.

^e Symbols: +, possesses property; 0, does not possess property.

than either adenovirus or SV40. The observed difference in kinetics of inactivation between MAC-7 and adenovirus 7 is not due to a difference in protein coats, because it has previously been shown (7, 8) that both members are encased in serologically similar adenovirus capsids. In a previous study with PARA-adenovirus 7 (6), the induction of SV40 tumor antigen was used as the marker, and it was concluded that the different virus capsids (adenovirus versus SV40) were not responsible for the different rates of inactivation observed for PARA and for complete SV40 upon exposure to UV light. Therefore, it can be assumed that the heterologous capsids are not the explanation for differences in sensitivity to inactivation by UV light between MAC and SV40 because MAC, like PARA, is encased in an adenovirus protein coat.

Neither PARA (12, 26, 30) nor MAC (8) induces the synthesis of SV40 viral, or capsid, antigen. However, PARA does induce the syn-

thesis of SV40 tumor antigen (12, 26, 30) and confers SV40 transplantation immunity in weanling hamsters (24, 27). As such, it is readily identified as defective SV40. MAC does not induce the synthesis of either of these antigens (8, 24). In addition, DNA extracted from GMK cells infected with PARA hybridized in vitro with SV40-specific RNA (28); DNA from GMK cells infected with MAC did not hybridize with SV40 complementary RNA.

Therefore, all available evidence indicates that MAC did not originate from SV40. Since the discovery that SV15 is able to enhance the replication of human adenoviruses in GMK cells (19), the possibility also exists that MAC is derived from a simian adenovirus. However, sera which reacted with three different groups of simian adenovirus tumor antigens failed to react with GMK cells infected with MAC. This problem is still under investigation.

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