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Variants of Defective Simian Papovavirus 40 (PARA) Characterized by Cytoplasmic Localization of Simian Papovavirus 40 Tumor Antigen

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Three isolates of PARA (particle aiding replication of adenovirus)-adenovirus 7 out of a total of 112 clonal progeny derived by two successive plaque purifications in green monkey kidney cells (GMK) were found to induce the synthesis of simian papovavirus 40 (SV 40) tumor (T) antigen in the cytoplasm of infected cells. The variant viruses induced plaque formation in human embryonic kidney cells which followed one-hit kinetics. In GMK cells, plaque formation followed two-hit kinetics which converted to first-order kinetics in the presence of additional helper adenovirus type 7. Analysis of plaque progeny from human cells showed that the progeny could replicate only in human cells, whereas progeny from monkey cells could multiply in both human and monkey cells. Heterologous human adenoviruses were able to enhance plaque formation by the variant viruses in monkey kidney cells. Neutralization tests indicated that both components of the populations had a type 7 adenovirus capsid. All three viruses were capable of inducing SV40 transplantation immunity in weanling hamsters. These results indicate the three variants are PARA-adenovirus 7 populations. Response of the induction of the synthesis of the cytoplasmic antigen to metabolic inhibitors was the same as for the synthesis of the nuclear SV40 T antigen. Different pools of sera which reacted with the intranuclear SV40 T antigen also detected the cytoplasmic antigen induced by the variant viruses. An adsorption experiment with cells containing either nuclear or cytoplasmic T antigen to remove tumor antibody from hamster sera also indicated that it is probably SV40 T antigen which is responsible for the cytoplasmic reaction. The species of the host cell—human, simian, or rabbit—appeared to play no role in the altered localization of this antigen. It is postulated that these PARA variants are further defective in some virus-mediated transport mechanism which shifts the T antigen from the cytoplasm to the nucleus.

Simian papovavirus (SV40) induces the synthesis of an antigen during the early stages of its replicative cycle in simian cells which can be detected using sera from hamsters bearing tumors induced by SV40 (8, 15, 21). This antigen, called the tumor or T antigen, is localized in the nucleus as particulate specks with the immunofluorescence technique (15).

A defective SV40 genome (PARA, particle aiding replication of adenovirus), found associated with a strain of adenovirus type 7 "adapted" to growth in green monkey kidney cells [GMK; 9, 16, 19] and encased in an adenovirus capsid, was recognized by the fact that it induced the synthesis of the intranuclear SV40 T antigen. Properties of the PARA-adenovirus 7 population, which has been analyzed in great detail, were recently reviewed by Rapp (Annu. Rev. Microbiol., *in press*).

In the study described in the preceding paper (17), progeny from 3 of 112 clones derived from the original PARA-adenovirus 7 stock by two successive plaque purifications in GMK cells induced the synthesis of an antigen in the cytoplasm of infected cells which reacted with hamster serum from SV40 tumor-bearing animals. There was no typical intranuclear SV40 T antigen reaction. This report will present evidence that these three variants are still PARA-adenovirus 7 populations and that the cytoplasmic reaction is due, in all probability, to the presence of SV40 T antigen in the cytoplasm.

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MATERIALS AND METHODS

Viruses. The history of the original PARA-adenovirus 7 (stock SP2) has been described previously (16). In this study, the virus was used after two additional passages in primary African GMK cells. The isolation of the three PARA variants to be described in this report was outlined in the preceding paper (17). They will be called PARA (1cT)-adenovirus 7, PARA (2cT)-adenovirus 7, and PARA (3cT)-adenovirus 7 and were used after four additional passages in GMK cells after the plaque purification procedure.

Adenovirus type 2 was a human isolate supplied by M. Benyesh-Melnick. It has been passed six times in KB cells and once in human embryonic kidney (HEK) cells. Adenovirus type 3 was obtained from B. A. Rubin (Wyeth Laboratories, Philadelphia, Pa.). It has been plaque-purified three times in HEK cells and then passed twice in the same cells. Adenovirus type 6 was the prototype strain obtained from the National Communicable Disease Center, Atlanta, and has been passed once in HEK cells and once in KB cells. Two strains of adenovirus type 7 were used. One was isolated from a fatal case of pneumonia (2) and has been passed three times in HEK cells and once in KB cells. The other strain was derived from the LL strain (16) by three successive plaque purifications in HEK cells (3) and has since been passed five times in the same cells. Adenovirus type 21 was obtained from the American Type Culture Collection (Rockville, Md.) and has been passed once in both HEK and KB cells. None of these adenoviruses induces the synthesis of SV40 T antigen and none can replicate in GMK cells in the absence of SV40 or PARA (14).

SV40 was the Baylor reference strain used in previous reports from this laboratory (15). It has been passed seven times in GMK cells, plaque-purified three times in CV-1 cells, and then passed seven more times in CV-1 cells.

All virus stocks were prepared in cells growing in 16-oz prescription bottles. At time of maximum cytopathic effect (CPE), the cells were disrupted into the medium by two alternate cycles of quick-freezing and thawing. The cell debris was removed by low-speed centrifugation; the supernatant fluids were dispensed in 1-ml amounts in glass ampoules; the ampoules were sealed; and the contents were quick-frozen and stored at −70 C.

Virus assays. Adenovirus titers were obtained by plaque formation in HEK cells grown in 35-mm plastic petri dishes (3). SV40 was titrated in GMK cells in 60-mm plastic petri dishes (7). PARA was also titered in GMK cells, but in the presence of enough helper human adenovirus to infect all the cells in the culture (5). After an adsorption period of 1 to 2 hr at 37 C, the infected cultures were overlaid with a mixture of Eagles medium, 10% fetal bovine serum, 0.23% $NaHCO_3$, 1% agar plus 100 units of penicillin and 100 μg of streptomycin per ml. The cultures were incubated at 37 C in an atmosphere of 5% CO_2. One week later, a second overlay containing a 1 to 20,000 dilution of neutral red was added. Adenovirus and PARA plaques were counted 10 to 14 days after inoculation and SV40 plaques were counted 14 to 18 days after inoculation of the virus. Immunofluorescence techniques were described in the accompanying paper (17).

Animal experiments. A transplantation rejection test was carried out by inoculating weanling hamsters three times at weekly intervals with virus, followed by challenge with hamster cells transformed by SV40 (H-50 cell line). The H-50 cells, derived originally from a hamster tumor induced by SV40 (1), were passed about 100 times in tissue culture and were then transplanted twice in hamsters to increase their transplantability; they were subsequently passed in tissue culture about 10 times. The H-50 cells contain SV40 T antigen (11), surface antigen (22), and viral deoxyribonucleic acid (DNA) (23), but do not yield infectious virus even after fusion with susceptible cells (10, 23).

The immunized hamsters were challenged by subcutaneous inoculation of 10-fold dilutions of H-50 cells, ranging from 10^2 to 10^5 cells per animal. Eight animals were inoculated with each concentration of cells. The number of cells required to produce tumors in 50% of the recipient hamsters (TPD_{50}) in each group was calculated by the method of Reed and Muench.

RESULTS

Appearance of cytoplasmic reaction induced by PARA variants. GMK cells infected with different PARA-adenovirus 7 populations were harvested 24 hr later, and were then treated with the immunofluorescence reagents described above. The typical intranuclear SV40 T antigen induced by parental PARA-adenovirus 7 is shown in Fig. 1a. In contrast, PARA variants 1cT, 2cT, and 3cT did not induce the synthesis of the intranuclear T antigen. Instead, a diffuse cytoplasmic reaction was observed (Fig. 1b).

Conditions for induction of cytoplasmic reaction. Experiments were then performed using GMK cells to determine if the induction of the cytoplasmic reaction responded to inhibitors and to antisera the same as the induction of the intranuclear T antigen by the parental PARA-adenovirus 7 or SV40. Cultures of GMK cells were infected with the three variants of PARA, parental PARA-adenovirus 7, SV40, or human adenovirus type 7. The cultures were harvested 24 hr after infection and stained for T antigens, whereas replicate cultures were harvested 48 hours after infection and stained for viral capsid antigens. The results are shown in Table 1.

The three variants of PARA induced the synthesis of the cytoplasmic reaction described above, whereas parental PARA-adenovirus 7 and complete SV40 induced the synthesis of intranuclear T antigen. When 10 μg of arabinofuranosylcytosine (ara-C) per ml was added to the cultures at the time of infection, no inhibition of synthesis of the cytoplasmic antigen or intra-

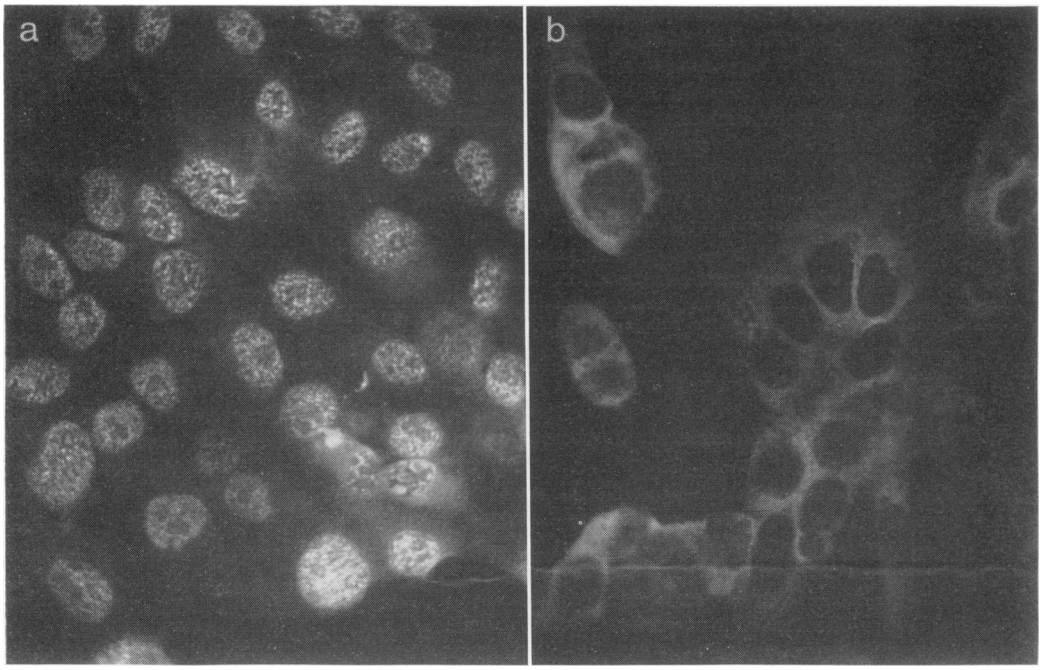

FIG. 1. *SV40 tumor antigen synthesized in GMK cells. (a) Induced by parental PARA-adenovirus 7 and localized in the nucleus; (b) induced by variant PARA (3cT)-adenovirus 7 and localized in the cytoplasm.*

TABLE 1. *Comparison of induction of cytoplasmic and intranuclear SV40 T antigen*

Treatment	Test system	Viruses					
		PARA (1cT)	PARA (2cT)	PARA (3cT)	PARA-Adeno 7	SV40	Adeno 7
None	SV40 T	cyt +[a]	cyt +	cyt +	nuc +[b]	nuc +	NT[c]
Ara-C	SV40 T	cyt +	cyt +	cyt +	nuc +	nuc +	NT
Cycloheximide	SV40 T	0[d]	0	0	0	0	NT
Anti-SV40	SV40 T	cyt +	cyt +	cyt +	nuc +	0	0
Anti-Adeno 7	SV40 T	0	0	0	0	nuc +	0
None	SV40 V	0	0	0	0	nuc +	0
None	Adeno T	nuc +	nuc +	nuc +	nuc +	NT	nuc +
Anti-SV40	Adeno T	nuc +	nuc +	nuc +	nuc +	0	NT
Anti-Adeno 7	Adeno T	0	0	0	0	0	0
None	Adeno V	nuc +	nuc +	nuc +	nuc +	NT	nuc +

[a] Cyt +, antigen localized in the cytoplasm.
[b] Nuc +, antigen localized in the nucleus.
[c] NT, not tested.
[d] Antigen not detected, 0.

nuclear T antigen was observed. However, the addition of 25 μg of cycloheximide per ml to replicate cultures at the time of infection inhibited the synthesis of both the cytoplasmic and intranuclear antigens. Pretreatment of the virus inocula with monkey serum containing neutralizing antibodies to SV40 did not affect the ability of the viruses to induce the respective reactions, with the exception of SV40 which was neutralized. Conversely, only SV40 was able to induce T antigen after the viruses were treated with adenovirus 7 antiserum. SV40 viral antigen was detected only in those cells which had been infected with complete SV40.

Replicate cultures were also stained for adenovirus antigens. The adenovirus T antigen was

detected in the nuclei of cells in cultures infected with an adenovirus. Treatment of the viruses with SV40 antiserum had no effect on the induction of the T antigen, but adenovirus antiserum inhibited induction of the antigens. Adenovirus viral antigen was also synthesized in the nuclei of cells in all cultures productively infected with an adenovirus.

Therefore, this experiment indicated that the development of the cytoplasmic reaction responded to inhibitors the same as the production of the intranuclear T antigen. It also appeared that the agent responsible for the induction of the cytoplasmic reaction was neutralized by adenovirus 7 antiserum, a fact already established for induction of the intranuclear T antigen by the parental PARA-adenovirus 7 (16, 19).

Evidence that the variant viruses are PARA-adenovirus populations. Kinetics of plaque formation by two of the variant viruses in HEK cells and in GMK cells in the presence and in the absence of helper adenovirus were determined. Figure 2 shows that plaque formation by both PARA (1cT) and PARA (3cT) followed one-hit kinetics in HEK cells. This is apparent because the titer of the stock, calculated from the number of plaques formed at sequential twofold dilutions of the virus, was the same when calculated from each dilution. This indicates that only one particle is required to initiate plaque formation in the human cells. These same viruses behaved differently in GMK cells (Fig. 3). Plaque formation followed two-hit kinetics, indicating a need for the interaction of two particles to form a plaque. This is evidenced by the fact the apparent undiluted titers fell rapidly as higher dilutions

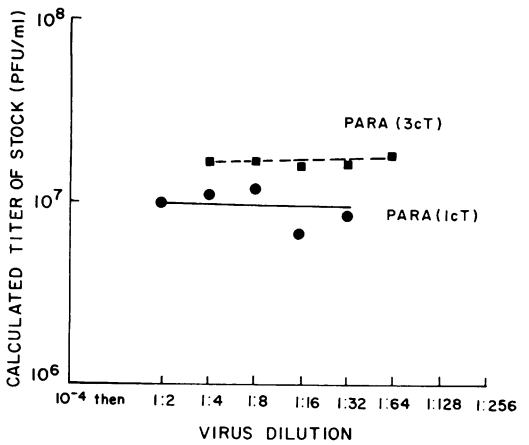

FIG. 2. *Kinetics of plaque formation in HEK cells by PARA (1cT)-adenovirus 7 and PARA (3cT)-adenovirus 7.*

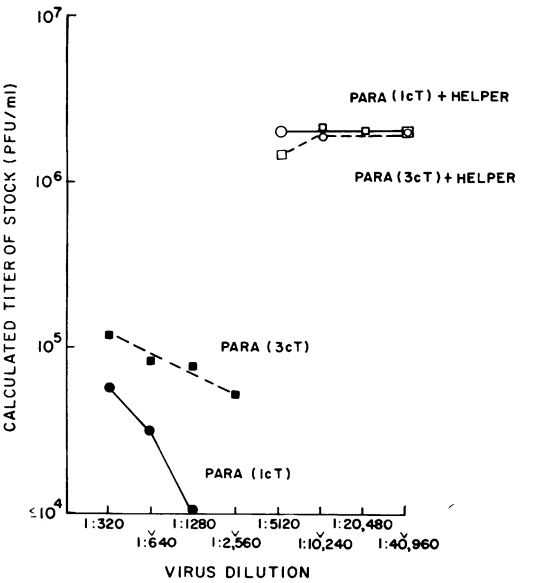

FIG. 3. *Kinetics of plaque formation in GMK cells by PARA (1cT)-adenovirus 7 and PARA (3cT)-adenovirus 7 in the presence and in the absence of helper human adenovirus type 7.*

of the viruses were used for calculations. In the presence of additional helper adenovirus type 7, assumed to be one of the two particles, plaque formation converted to one-hit kinetics. It can also be seen in Fig. 3 that the calculated titer of the PARA-adenovirus stocks was higher in the presence of helper adenovirus. These kinetics of plaque formation are identical to those described for the parental PARA-adenovirus 7 population (4).

Plaques which had been initiated in HEK and GMK cells by the three variant viruses were picked and their host range determined (Table 2). Ten plaques were studied for each virus from each cell type. Without exception, plaques which were picked from HEK cells yielded progeny which were able to induce progressive CPE only in HEK cells but not in GMK cells. However, plaques which originated from GMK cells yielded progeny which were able to cause the development of CPE in both HEK and GMK cells. These results are the same as those reported earlier for parental PARA-adenovirus 7 (3, 12, 20), and indicate a mixed population consisting of (i) human adenovirus which replicates in HEK cells and (ii) PARA which multiplies in GMK cells in the presence of a helper adenovirus. The same plaque progeny are currently being tested for ability to induce the synthesis of the cytoplasmic reaction.

TABLE 2. *Host range of progeny from plaques formed in HEK[a] and GMK[b] cells*

Parental virus	Source of plaques	Cytopathic effects	
		HEK cells	GMK cells
PARA (1cT)	HEK	10/10[b]	0/10
PARA (2cT)	HEK	10/10	0/10
PARA (3cT)	HEK	10/10	0/10
PARA (1cT)	GMK	10/10	10/10
PARA (2cT)	GMK	10/10	10/10
PARA (3cT)	GMK	10/10	10/10

[a] HEK, human embryonic kidney; GMK, green monkey kidney.

[b] Numerator, number of plaques whose progeny induced cytopathic effects; denominator, number of plaques tested.

The addition of excess amounts of human adenovirus type 7 to the GMK cell assay plates increased the plaque-forming capacity of two of the variant viruses (Fig. 3). Similar experiments were performed to determine the effect of the addition of heterologous human adenovirus types 2, 3, 6, and 21 on plaque formation by the PARA (cT) mutants. The plaque titer of each of the variant viruses in the presence and absence of the helper adenoviruses is shown in Table 3. The enhancement of the plaque titer in the presence of a given helper virus over that of the control (no helper virus) is shown in parentheses. In general, the heterologous helper viruses enhanced the plaque titer of the PARA (cT) mutants 10-fold or greater. Plaque formation by PARA (3cT) was not enhanced as much as for the other two variants and this may be due to the fact the PARA (3cT) stock contained a slightly higher titer of adenoviruses (Table 4). Enhancement of plaque formation by heterologous helper adenoviruses was also routinely obtained with the parental PARA-adenovirus 7 (6, 14). Enhanced plaques which developed beyond the end point obtained in the absence of additional helper virus have been picked from plates inoculated with all three variant populations. They will be studied to determine if "transcapsidation" (12) has occurred and if the localization of the cytoplasmic reaction is helper-dependent.

Data in Table 1 suggested that the variant virus populations were antigenically type 7 adenoviruses and this was confirmed by neutralization of plaque formation in HEK and GMK cells (Table 4). The HEK cells were used to detect the adenovirus component of the population. It can be seen that with all three variant populations, antiserum against SV40 had no effect on plaque formation in HEK cells, whereas anti-adenovirus 7 serum inhibited plaque formation. Similar neutralization tests, with one modification, were performed in GMK cells to detect the PARA component of the populations. Heterologous helper adenovirus type 2 was used as a helper virus for PARA to circumvent the neutralization of the helper adenovirus 7 by the antiserum. However, even in the presence of heterologous helper virus, plaque formation by PARA was inhibited by adenovirus 7 antiserum. Therefore, this experiment showed that both components of all three variant populations were encased in adenovirus 7 capsids. This type of neutralization test had been used to demonstrate the antigenicity of both components of the parental PARA-adenovirus 7 stock (6, 12).

The parental PARA-adenovirus 7 was known to induce SV40 transplantation immunity in weanling hamsters (13, 18). A transplantation rejection test was, therefore, performed to determine if the three variant populations also carry this SV40 marker (Table 5). The H-50 cells were used as challenge cells. The history and properties of this cell line are outlined above. As summarized in Table 5, all three variant PARA-

TABLE 3. *Enhancement of plaque formation of variant PARA-adenovirus 7 populations in simian cells by heterologous human adenoviruses*

Helper virus	Plaque-forming units per ml		
	PARA (1cT)	PARA (2cT)	PARA (3cT)
None	8.3×10^4	3.8×10^4	2.5×10^5
Adenovirus 2	2.0×10^6 (24)[a]	7.0×10^5 (18)	2.3×10^6 (9)
Adenovirus 3	3.3×10^6 (40)	1.8×10^6 (47)	6.5×10^5 (2.6)
Adenovirus 6	2.5×10^6 (30)	8.0×10^5 (21)	2.2×10^6 (9)
Adenovirus 21	6.5×10^5 (8)	4.0×10^5 (10)	3.5×10^5 (<2)

[a] Numbers in parentheses indicate fold enhancement in plaque titer over control with no helper virus added.

TABLE 4. *Neutralization of plaque formation by variant PARA-adenovirus 7 populations*

Treatment	Plaque forming units per ml					
	PARA (1cT)		PARA (2cT)		PARA (3cT)	
	HEK	GMK[a]	HEK	GMK[a]	HEK	GMK[a]
Normal serum	1.0×10^7	2.0×10^6	6.0×10^6	7.0×10^5	2.2×10^7	2.3×10^6
Anti-SV40 serum	2.0×10^7	2.9×10^6	1.6×10^7	1.4×10^6	2.6×10^7	2.2×10^6
Anti-adenovirus 7 serum	$<1 \times 10^3$	$<1 \times 10^3$	$<1 \times 10^3$	$<1 \times 10^3$	$<1 \times 10^3$	$<1 \times 10^3$

[a] Adenovirus type 2 present as helper virus.

TABLE 5. *Induction of SV40 transplantation resistance by variant PARA-adenovirus 7 populations*

Virus immunization	12-weeks postchallenge		SV40 transplantation immunity
	TPD_{50}[a]	Resistance index	
None	<100		
PARA (1cT)	1,000	>10	yes
PARA (2cT)	20,000	>200	yes
PARA (3cT)	13,000	>130	yes

[a] TPD_{50}, number of H-50 challenge cells required to produce tumors in 50% of the recipient animals.

adenovirus 7 populations immunized the weanling hamsters so that increased numbers of H-50 cells were required to produce tumors. The resistance index was calculated by dividing the TPD_{50} of an immunized group by the TPD_{50} of the control, nonimmunized group. Values greater than 10, 130, and 200 were obtained with the 3 virus populations. A resistance index of 10 or more is considered to be indicative of transplantation immunity. Therefore, all three variant PARA-adenovirus 7 populations carry the marker for SV40 transplantation immunity. It had previously been shown that the inoculation of adenovirus 7 does not induce resistance to the H-50 cells (13).

Influence of host cell on localization of SV40 T antigen. A variety of different species of animals were surveyed to determine if the origin of the host cell influenced the localization of SV40 T antigen. The three PARA (cT) variants were compared with parental PARA-adenovirus 7 in an immunofluorescence test to detect SV40 T antigen. Primary GMK cells, three stable cell lines derived from GMK cells (VERO, CV-1, and BSC-1), HEK cells, primary rabbit kidney cells (Rab K), and a stable cell line derived from Rab K cells (RK-13) were used. The host cell apparently played no role in the altered localization of the T antigen induced by the PARA (cT) variants (Table 6). It should be noted that with the same inoculum, many fewer positive cells were observed in the VERO, CV-1, and BSC-1 cells, and only rare positive cells were observed in the cultures of RK-13 cells. The cytoplasmic localization of T antigen in cells infected by the variants of PARA, therefore, appears to be a property of the viruses and not of the host cell.

Evidence that the cytoplasmic antigen is SV40 T antigen. All the evidence in the preceding section indicated that the three variant viruses were still PARA-adenovirus 7 populations. The remaining question to be considered was whether the reaction detected in the cytoplasm of infected cells was actually due to the presence of SV40 tumor antigen. The induction of the cytoplasmic antigen responded to inhibitors the same as the induction of the intranuclear SV40 T antigen (Table 1).

A variety of different sera were tested by immunofluorescence to determine if the ability

TABLE 6. *Influence of host cell on localization of SV40 T antigen induced by PARA-adenovirus variants*

Host cell[a]	Virus			
	PARA (1cT)	PARA (2cT)	PARA (3cT)	PARA-adeno 7
GMK	cyt +[b]	cyt +	cyt +	nuc +[c]
VERO	cyt +	cyt +	cyt +	nuc +
CV-1	cyt +	cyt +	cyt +	nuc +
BSC-1	cyt +	cyt +	cyt +	nuc +
HEK	cyt +	cyt +	cyt +	nuc +
Rab K	cyt +	cyt +	cyt +	nuc +
RK-13	cyt +	cyt +	cyt +	nuc +

[a] GMK, green monkey kidney; VERO, CV-1, and BSC-1, stable cell lines derived from GMK; HEK, human embryonic kidney; Rab K, rabbit kidney; RK-13, stable cell line derived from Rab K.

[b] Cyt+, antigen localized in the cytoplasm.

[c] Nuc +, antigen localized in the nucleus.

to react with the intranuclear SV40 T antigen correlated favorably with the ability to detect the cytoplasmic reaction induced by PARA (2cT)-adenovirus 7 in GMK cells (Table 7). Six different pools of sera from hamsters bearing transplants of H-50 cells (derived from a tumor induced by SV40), plus a pool of hamster sera from animals bearing another line of SV40-tumor transplants (obtained from David Porter), detected the cytoplasmic antigen as well as the nuclear T antigen. Individual sera from hamsters with tumors induced by either PARA-adenovirus 12 or PARA-adenovirus 7 reacted with both the intranuclear and cytoplasmic antigens. The serum from an animal with a BHK-21 cell tumor did not react in either test. Significantly, a pool of serum from three rabbits immunized with a high dose of purified SV40 and shown to contain antibodies against SV40 T antigen (S. S. Tevethia, *unpublished data*) also detected the cytoplasmic reaction. The reaction detected by the rabbit serum is shown in Fig. 4 and can not be distinguished from that detected using hamster serum (Fig. 1b).

TABLE 7. *Survey of different sera for ability to detect the cytoplasmic reaction induced by PARA (2cT)-adenovirus 7 in simian cells*

Serum	Source of serum	Ability to detect	
		Nuclear T antigen	Cytoplasmic antigen
Pool 20	Hamsters with SV40-tumor transplants (H-50)	+[a]	+
Lot 95	Hamsters with SV40-tumor transplants (H-50)	+	+
Lot 96	Hamsters with SV40-tumor transplants (H-50)	+	+
Lot 97	Hamsters with SV40-tumor transplants (H-50)	+	+
Lot 98	Hamsters with SV40-tumor transplants (H-50)	+	+
Lot 110	Hamsters with SV40-tumor transplants (H-50)	+	+
P-SV40	Hamsters with SV40-tumor transplants	+	+
PA-12	Hamster with a tumor induced by PARA-adenovirus 12	+	+
739-R	Hamster with a tumor induced by PARA-adenovirus 7	+	+
1059-R	Hamster with a tumor induced by PARA-adenovirus 7	+	+
870-0	Hamster with a tumor induced by PARA-adenovirus 7	+	+
1192-RL	Hamster with a tumor induced by PARA-adenovirus 7	+	+
942RL	Hamster with a tumor induced by PARA-adenovirus 7	+	+
D-3	Hamster with tumor induced by BHK-21 cells	0[b]	0
Day 68	Rabbits immunized with purified SV40	+	+

[a] Reaction detected, +.
[b] No reaction, 0.

An adsorption experiment was performed to determine if the antibody against the intranuclear SV40 T antigen was removed in parallel with the antibody which reacted with the cytoplasmic antigen. The following cells were used to adsorb the serum: GMK (control) cells, GMK cells infected with parental PARA-adenovirus 7 and demonstrating approximately 50% CPE, BHK-21 hamster cells, H-50 hamster tumor cells, and H-2cT-1, a clonal cell line derived from hamster embryo cells transformed in vitro by PARA (2cT)-adenovirus 7 and shown to contain SV40 T antigen in the cytoplasm of most of the cells (Duff, Butel, Richardson, and Rapp, *unpublished data*). The serum used in the adsorption experiment was lot 96, a pool of serum from hamsters bearing SV40 tumor transplants and known to react with the intranuclear SV40 T and the PARA (cT) cytoplasmic reactions. Samples of the serum were adsorbed in the following manner. The different types of cells were scraped from bottles, pelleted in test-tubes, and washed with tris(hydroxymethyl)aminomethane (Tris) buffer. Each type of cell yielded about a 0.4-ml volume of cell pellet. Lot 96 serum (0.4 ml), diluted near the immunofluorescence end point (1:16), was added to each pellet. The cell pellets were then frozen and thawed three times into the serum, the mixtures were incubated for 30 min at 37 C, and the cell debris was removed by low-speed centrifugation. The adsorbed serum was then tested for its ability to detect the cytoplasmic reaction induced in GMK cells by PARA (2cT)-adenovirus 7, the intranuclear SV40 T antigen in the H-50 cells, and the cytoplasmic antigen in the H-2cT-1 cells (Table 8). Adsorption of the serum with control GMK and BHK-21 cells did not affect the staining reactions. Adsorption of the serum with GMK + PARA-adenovirus 7 or with the H-2cT-1 cells completely removed the antibodies which reacted in all three test systems. Adsorp-

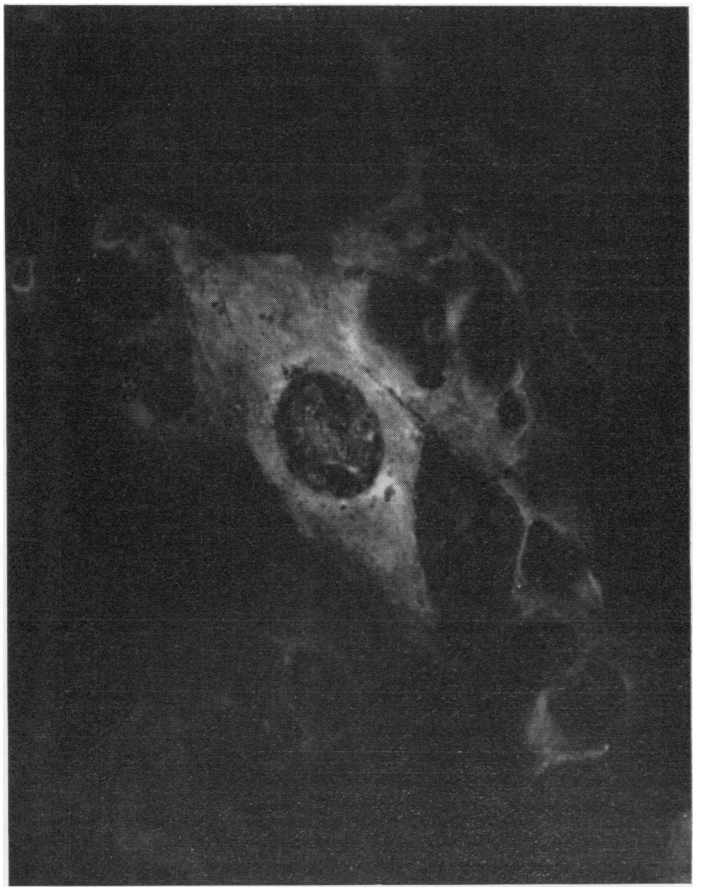

FIG. 4. *SV40 tumor antigen induced by PARA (2cT)-adenovirus 7 in GMK cells and detected with sera from rabbits immunized with partially purified SV40.*

TABLE 8. *Adsorption of serum containing SV40 tumor antibody*

Serum adsorbed with	Ability of adsorbed serum to react with		
	GMK + PARA (2cT)	H-50	H-2cT-1
None	+	+	+
GMK control	+	+	+
GMK + PARA-adenovirus 7	0	0	0
BHK-21	+	+	+
H-50	±	±	±
H-2cT-1	0	0	0

tion with the H-50 cells greatly diminished the reaction in each test system but did not completely remove it. It is important to notice that, in each case, the antibody which reacted with the intranuclear T antigen and the antibody which reacted with the cytoplasmic antigen in both productively-infected and transformed cells were removed in parallel. In addition, cells containing either the intranuclear T antigen (GMK + PARA-adenovirus 7 and H-50) or the cytoplasmic antigen (H-2cT-1) removed the antibodies from the serum. Therefore, this experiment indicates that the cytoplasmic antigen is serologically similar, or identical, to SV40 T antigen.

DISCUSSION

This paper reports the isolation by plaque-purification in GMK cells of three variants of PARA-adenovirus 7 which induce the synthesis of SV40 T antigen in the cytoplasm of infected cells. A variety of tests were carried out to determine whether the variant viruses were still PARA-adenovirus 7 populations. These tests included determination of kinetics of plaque formation in HEK and GMK cells, analysis of

plaque progeny from HEK and GMK cells, enhancement tests with heterologous helper adenoviruses, neutralization tests, and a transplantation rejection test using weanling hamsters. All of these tests indicated that the three variants were, indeed, PARA-adenovirus 7 populations.

Experiments were then designed to determine if the cytoplasmic reaction was actually due to the presence of SV40 T antigen. A variety of different serum pools were tested and all those which reacted with intranuclear SV40 T antigen were capable of detecting the cytoplasmic antigen. An adsorption experiment showed that the antibody (or antibodies) which detected the nuclear and cytoplasmic antigens was (were) removed in parallel. Immunological identity of purified antigens has not yet been demonstrated and tumors have not yet been produced in hamsters by the variant viruses so antiserum against the cytoplasmic antigen has not become available to test against the intranuclear T antigen. However, all evidence obtained to date indicates that it is SV40 T antigen which is responsible for the cytoplasmic reaction.

Several different host cells (human, simian, rabbit) were surveyed and none appeared to exert any influence on the localization of the respective reactions induced by parental PARA-adenovirus 7 or the PARA (cT) variants. One must conclude, then, that this is due to a property of the viruses. One explanation might be that there is a virus-mediated transport mechanism involved in the transfer of SV40 T antigen from the cytoplasm, where it is theoretically synthesized, into the nucleus. These three variants would, therefore, have lost the function of inducing this virus-mediated transfer. This concept is strengthened by the fact that hamster cells transformed in tissue culture by one of the variants also contain SV40 T antigen in the cytoplasm (Duff, Butel, Richardson and Rapp, *unpublished data*) in contrast to other SV40 or PARA-transformed cell lines which contain T antigen in the nucleus. Other explanations for this altered localization of the T antigen may become apparent as the viruses are studied in more detail.

It should be stressed that these three variants are capable of completing their replicative cycles with the production of infectious progeny and the resulting progeny also induce the synthesis of SV40 T antigen in the cytoplasm of cells. Therefore, whatever effects the altered localization of the tumor antigen may have, it is not a lethal effect. The effect of the altered localization of the T antigen on the replicative and transforming cycles of the viruses is currently under investigation.

There is not yet enough evidence to indicate whether the three variants described in this paper are the same or different. Since they were isolated from a single stock of parental PARA-adenovirus 7, it is possible that all three are progeny of a single mutant virus particle.

It should be mentioned that the cytoplasmic localization of the SV40 T antigen may not be an entirely clear-cut phenomenon. When infected cells are stained by immunofluorescence, the nuclei of positive cells are not always completely black. No particulate stippling, typical of the intranuclear SV40 T antigen, has ever been observed but sometimes there is a suggestion of an ill-defined reaction. Such reactions may be due to antigen in the cytoplasm which superimposed over the nucleus. It is also possible, however, that in some cells a small amount of the antigen may leak into the nucleus even though the postulated transport mechanism is not functioning efficiently. The observations reported would support the concept that the SV40 T antigen is synthesized in the cytoplasm of infected cells and is then transferred to the nucleus.

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