*STUDIES OF ADENOVIRUS-SV40 HYBRID VIRUSES, V. EVIDENCE FOR LINKAGE BETWEEN ADENOVIRUS AND SV40 GENETIC MATERIALS**

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Communicated by Robert J. Huebner, March 21, 1966

Recent studies have established that the adenovirus type 7 (Ad. 7) strain E46+ is a mixed population of infectious adenovirus particles and defective particles consisting of adenovirus capsids containing the segment of SV40 DNA which codes for T antigen.[1-4] Because of the inability to obtain the SV40 DNA carriers as a pure population, it has not been possible to determine whether they also carry adenovirus genetic material and whether there is interaction between the two DNA's at the molecular level. The observation that the SV40 DNA could be transferred from E46+ to other adenovirus types[5, 6] provided a technique for testing for such linkage. This report describes transfer of an Ad. 7 gene with the SV40 gene, with evidence that they are present in the same capsid.

Materials and Methods.—Viruses: The substrains of the prototype Ad. 2 and Ad. 12 strains which had acquired the SV40 gene by mixed infection with E46+ have been described previously;[5] they will be referred to here as Ad. $2^{+(t7)}$ and Ad. $12^{+(t7)}$ to indicate that they acquired the SV40 gene by transfer from Ad. 7. The pool (pool 1017) of Ad. $2^{+(t7)}$ used previously[5] was employed for most of the present studies. It represented the tenth African green monkey kidney (AGMK) tissue culture passage after mixture with E46+; passages six through eight were made with Ad. 7 rabbit antiserum, and the ninth was made by limiting dilution passage.

The passage history of the Ad. $12^{+(t7)}$ virus used here was as follows. Prototype Ad. 12 (Huie) was mixed with E46+ and inoculated into AGMK cultures. The harvest was passed once in AGMK with Ad. 7 rabbit serum, then twice in human embryonic kidney (HEK) cultures with the Ad. 7 antiserum; a plaque was then isolated in AGMK cells, and this was passed four more times in AGMK. Both virus strains were free of detectable Ad. 7 virus as determined by breakthrough neutralization tests.

Plaque isolates were obtained in AGMK cultures, using the procedure described previously.[4]

Standard rabbit antisera against prototype adenovirus strains were used for neutralization tests.

Hamster antisera: The majority of tests for Ad. 7 T antigen were done with a serum pool (pool C) from hamsters carrying transplanted tumors induced by the Pinckney strain of Ad. 7; this strain does not carry the SV40 genetic material. The sera included in this pool were selected for having high-titer (>1:160) complement-fixing (CF) antibody against early cell pack antigens[7] of cells infected with Ad. 7. The pool reacted in CF with Ad. 7 tumor extract to a titer of 1:20, and was negative against standard Ad. 7 viral antigen at 1:10 serum dilution. An additional serum pool and three individual sera from hamsters carrying Ad. 7 (nonhybrid) tumors were used for confirmatory tests; these sera were selected on the basis of reacting in CF with early cell pack antigens of Ad. 7 and/or Ad. 3 and staining intranuclear antigens in cells infected with Ad. 7 in the fluorescent antibody (FA) test. The patterns of immunofluorescent reactivity of the Ad. 7 hamster sera are described below.

Pooled serum from hamsters carrying transplanted Ad. 12 tumors was used as a group-reactive immunofluorescent reagent for estimating the total amount of infectious adenovirus.[8] Although reactive only with the Ad. 12, 18, 31 subgroup in the CF test, Ad. 12-tumored hamster sera are much more broadly reactive in the FA test. They consistently stain intranuclear antigens in cells infected with Ad. 7 strains,[8, 9] but only a few (5 of 16 sera individually tested) stain cells infected with Ad. 2. The serum pool used in the present studies (pool B) stained cells infected with Ad. 1 through 18.

SV40 T antigen was stained with the SV40-tumored hamster serum pool used in previous studies.[2, 4, 5]

Immunofluorescent assays: Tests for immunofluorescent stainable antigens were done in HEK cells as previously described.[8, 10] Infected cells were generally fixed 24 hr after inoculation; in a few tests 48-hr fixation was used. All fluorescent antibody (FA) tests were done by the indirect procedure, using the hamster sera at a dilution of 1:10.

Results.—Serologic reactivity of Ad. 7-tumored hamster serum pool: To attempt to demonstrate transfer of Ad. 7 genetic material, serological markers of Ad. 7 were studied. It did not seem fruitful to study capsid antigens since Ad. $2^{+(t7)}$ was not inhibited by Ad. 7 rabbit antiserum,[5] and hypothetical hybrids with respect to capsid antigen induction would probably have been eliminated by the early passages with Ad. 7 antiserum. Instead, emphasis was placed on finding T antigens of Ad. 7 not shared by Ad. 2 or Ad. 12, and testing for their induction by the transfer hybrids.

Two possible T antigen markers were found. As mentioned above, most FA positive sera from Ad. 12-tumored hamsters react with an Ad. 7 antigen in acutely infected cells, but not with Ad. 2. On the presumption that this antigen of Ad. 7 might reflect a gene not present in Ad. 2, tests were made to determine if Ad. $2^{+(t7)}$ induced, in HEK cells, an antigen stainable with Ad. 12 hamster sera which did not stain the parent Ad. 2. No staining was obtained.

The second marker studied was the antigen(s) stainable with Ad. 7-tumored hamster serum pool C. When tested against HEK cells infected with various Ad. 7 strains, this serum stained intranuclear antigen(s) in the form of short flecks or dots; the reactive antigens were formed equally well in Ad. 7-infected cells treated with 5-fluorodeoxyuridine (FUDR) ($10^{-4.7}$ *M* in thymidine-free medium, added 1 hr before infection) as in untreated cells. Resistance to FUDR inhibition is characteristic of early adenovirus antigens, whereas structural antigens, which appear later, are inhibited.[8, 11] The serologic reactivity of this serum in FA tests is summarized in Table 1. Pool C gave bright staining with those adenovirus types with which Ad. 7 shares complement fixation (CF) reactive T antigens[7] (types 3, 4, 11, 14, and 21) and with types 8, 10, and 13, but did not react at all with cells infected with viruses of the Ad. 1, 2, and 5 subgroup or with Ad. 12 or 18. Also, pool C did not stain cells infected with Ad. 2-SV40 hybrid strains produced by passage with SV40 or Ad. 1^{+}.[12, 13]

The pool did not react with Ad. 7 tumor cells; this is not unique, however, since only rare sera from hamsters with Ad. 7 tumors have given such reaction.[14]

Induction of Ad. 7 hamster serum-stainable antigens by Ad. $2^{+(t7)}$ and Ad. $12^{+(t7)}$: In contrast to the nonhybrid parent Ad. 2 and Ad. 12 viruses, the Ad. $2^{+(t7)}$ and Ad. $12^{+(t7)}$ viruses induced antigen(s) stainable by pool C (Table 1), the induction of this antigen being prevented by homologous rabbit antiserum but not by Ad. 7 antiserum. Table 2 shows results of neutralization tests and typifies the quantitative relationships of the percentage of cells showing the various antigens. The proportion of cells stained by pool C was generally three- to eightfold lower than that with Ad. 12 or SV40 hamster antisera; this probably reflects low efficiency of formation of the antigen rather than a smaller number of particles carrying the determinant, since a ratio of 2.5 of Ad. 12/Ad. 7 staining per cent was found with the Pinckney strain of Ad. 7 (Table 1); also, the cloning studies presented below in-

TABLE 1

IMMUNOFLUORESCENT REACTIVITY OF STANDARD POOLS OF TUMORED HAMSTER SERUM

Virus	Strain	Description	Percentage of HEK Cells Stained* Ad. 7 HS pool C	Ad. 12 HS pool B	SV40 HS
Ad. 7	Pinckney	Oncogenic field strain	8	20	
	14500	" " "	70	90	
	E46$^-$	Nonhybrid from E46$^+$ (ref. 9)	80		
Ad. 2	Ad. #6	Prototype	0†	98	
	Ad. 2$^-$	Nonhybrid from Ad. 2$^{+(t7)}$	0	99	
	Ad. 2$^{+(t1)}$	SV40 transfer hybrid from Ad. 1$^+$ (refs. 12, 14)	0		80
	Ind. 2	Ad. 2 hybrid with complete SV40 genome[13]	0		47
	Ad. 2$^+$	SV40 T antigen hybrid clone from Ind. 2[17]	0		70
	19325	Field strain	0	90	
	Ad. 2$^{+(t7)}$		3.3	42	17
	Ad. 2$^{+(t3)}$	SV40 transfer hybrid from Ad. 3$^+$ (refs. 12, 14)	15		60
Ad. 12	Huie	Prototype	0	90	
	Ad. 12$^{+(t7)}$		1.2	19	2.8
Ad. 1	Prototype strains		0	0.5‡	
3	"		100	99	
4	"		15	20	
5	"		0	1‡	
8	"		50	40	
10	"		5	8	
11	"		70	70	
13	"		5	10	
14	"		60	70	
18	"		0	70	
21	"		60	70	
SV40§			0		15

* HEK cells infected with various doses of the viruses. The percentage staining with Ad. 12 or SV40 hamster serum (HS) can be taken as a measure of the total infection rate.
† 0 = <0.01%.
‡ Dull staining.
§ Test done in AGMK cells.

TABLE 2

NEUTRALIZATION OF PARTICLES CONTAINING AD. 7 DETERMINANT BY ANTISERUM TO RECIPIENT SEROTYPE

Neutralization Mixture: Virus	Neutralization Mixture: Serum (1:20)	Percentage of Cells Stained in FA Test: Ad. 7 HS*	Ad. 12 HS	SV40 HS
Ad. 2$^{+(t7)}$	Normal RS	7		50
	Ad. 7 RS	5	20	30
	Ad. 2 RS	0†	0	0.01
Ad. 12$^{+(t7)}$	Normal RS	0.8	12	1.7
	Ad. 7 RS	0.9	15	1.7
	Ad. 12 RS	0	0	0

* HS, hamster serum; RS, rabbit serum.
† 0 = <0.05%.

dicate that all hybrid particles carry the determinant. The pattern of staining resembled that in Ad. 7-infected cells stained with pool C; staining was restricted to the nucleus and ranged from a few small dots of antigen in some nuclei to brilliant masses of balls or short stubby rods in others (Fig. 1). Induction of this antigen by Ad. 2$^{+(t7)}$ was not prevented by FUDR treatment of the cells, using the procedure mentioned above.

FA staining of Ad. 2$^{+(t7)}$ by Ad. 7 hamster serum was markedly diminished by mixture of the serum with an extract of hamster kidney tissue culture cells infected with Ad. 7, but was not blocked by mixture with a similar extract of cells infected with Ad. 2.

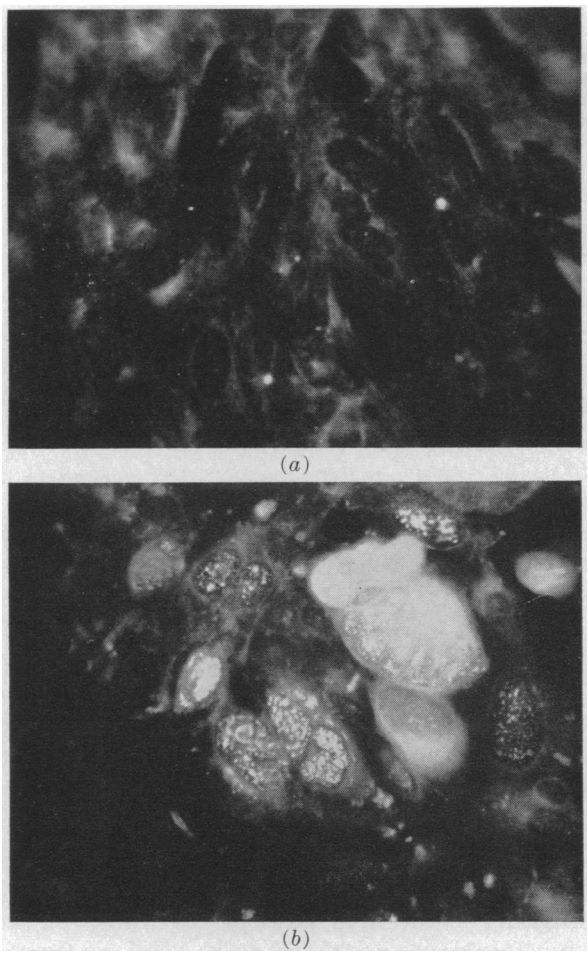

FIG. 1.—Immunofluorescent staining by Ad. 7-tumored hamster serum of HEK cells infected with Ad. 12 and Ad. $12^{+(t7)}$. 48-Hr harvests. (*a*) Ad. 12 infection. There is no nuclear staining. (*b*) Ad. $12^{+(t7)}$ infection. A number of nuclei show brilliant staining of dots and flecks.

The acquisition of ability to induce the Ad. 7 antigen was not unique to the two transfer experiments from which Ad. $2^{+(t7)}$ and Ad. $12^{+(t7)}$ derived; in each of three transfer experiments with $E46^+$ donor and Ad. 2 recipient and two such experiments with Ad. 12 recipient, the recipient type acquired both SV40 and Ad. 7 antigen-inducing capacity. In one of these experiments a line of $E46^+$ was used which had been freed of the adeno-associated virus (AAV),[15, 16] indicating that this common contaminant of adenovirus stocks was not participating in the transfer. Also, one of the tests was done in HEK cells; thus, monkey cells were not necessary for the transfer event.

Comparable results were obtained when Ad. 3^+ strain JF^{12} was used as donor and Ad. 2 as recipient; the Ad. 2 acquired the capacity to induce antigens stainable with SV40 hamster serum and Ad. 7 hamster serum pool C (Table 1).

TABLE 3

SEROLOGIC REACTIVITY OF ADDITIONAL AD. 7 HAMSTER SERA AND THEIR USE TO DEMONSTRATE TRANSFERRED GENETIC MATERIAL IN AD. $2^{+(t7)}$ AND AD. $12^{+(t7)}$

		Percentage of HEK Cells Stained				
		Ad. 7 Hamster Sera				Ad. 12 HS
Virus	Strain	Pool D*	#55612†	#2141-1-5*	#2141-2-1*	Pool B
Ad. 7	E46$^-$	90	90	90	90	95
Ad. 2	Ad. #6	0	0	0	0	50
	Ad. $2^{+(t7)}$	2.7	1.8	1.5	1.3	
Ad. 12	Huie	0	0	0	0	90
	Ad. $12^{+(t7)}$	1.4	0.5	0.8	0.2	20
Ad. 1	Prototypes‡	0	0	0	0	0.5
3	"	95	80	80	0	99
4	"	0	0	0	0	20
8	"	0	0	0	0	40
10	"	0	0	0	0	8
11	"	20	0	0	0	70

* Sera from hamsters carrying transplants of tumors induced by Pinckney strain of Ad. 7.
† Serum of a hamster carrying a primary tumor induced by Gomen strain of Ad. 7.
‡ These coverslips are from the same sets used in Table 1.

The induction of an Ad. 7 antigen by Ad. $2^{+(t7)}$ and Ad. $12^{+(t7)}$ was also demonstrable with Ad. 7 hamster sera other than pool C (Table 3). These sera stained cells infected with Ad. 7, Ad. $2^{+(t7)}$, and Ad. $12^{+(t7)}$, but gave no staining with Ad. 2 or Ad. 12. They were less reactive with heterologous adenovirus types than was pool C, and one serum (#2141-2-1) appeared to be type-specific.

Evidence for association of SV40 and Ad. 7 genetic material: The above findings establish that Ad. 7 genetic material is present in the transfer recipient preparations, in capsids of the recipient serotype. Three types of evidence indicate that it is present in the same capsids as the SV40 genetic material. First, 26 plaque isolates of Ad. $2^{+(t7)}$ and three plaque isolates of Ad. $12^{+(t7)}$ were obtained in AGMK cells and passed once in AGMK; all 29 induced both SV40 and Ad. 7 T antigen when inoculated into HEK cells. This finding alone does not prove that the two genetic materials are in the same particle, since plaque production by Ad.-SV40 hybrids is a two-hit process in AGMK cells[4, 5] and the titrations from which the 29 plaques were obtained showed typical 2-hit dose-response relations. Plaque production in this system requires dual infection by a particle with the complete adenovirus genome and a defective particle carrying the SV40 genome. Thus, it was necessary to demonstrate that the Ad. 7 genetic material was in defective particles to infer that it is in the SV40 carrying particle. This was tested by the progeny ratio method described previously[4, 5] for demonstration of defectiveness of the SV40 carriers in Ad.-SV40 hybrids. Ad. $2^{+(t7)}$ was titrated in tube cultures of HEK and AGMK cells, and the fluids and cells at each dilution were harvested at complete CPE; the titer in HEK was $10^{6.8}$, and in AGMK, $10^{4.1}$ tissue culture infectious doses ($TCID_{50}$) per 0.1 ml. The harvests were diluted 1:3, and 0.2 ml inoculated into HEK cells on coverslips. These were fixed at 24 hr and stained with Ad. 12, Ad. 7, SV40-tumored hamster sera. The results are shown in Figure 2. In AGMK cells, the three genetic determinants were produced in comparable proportions at all multiplicities, but in HEK cells, production of the Ad. 7 and SV40 determinants declined in proportion to the decrease in multiplicity after the multiplicity fell below one. The same type of result was observed in a similar test with Ad. $12^{+(t7)}$. This pattern establishes that the Ad. 7 gene carriers are defective,

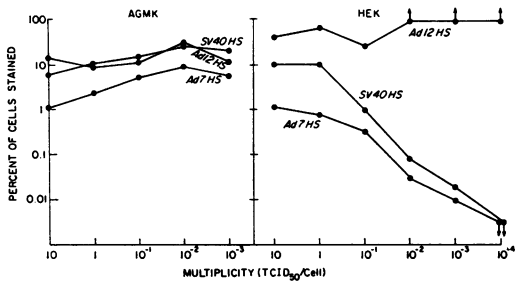

FIG. 2.—Antigen induction in HEK cells by progeny of titrations of Ad. $2^{+(t7)}$ in AGMK and HEK cells. Cultures were infected at the multiplicities shown on the abscissa, harvested at complete CPE, and the fluids inoculated at a dilution of 1:3 into HEK coverslip cultures. The coverslips were fixed at 24 hr, and the percentage of cells showing antigens stainable with the various tumored hamster sera (HS) was determined.

and together with the cloning data indicates that the SV40 and Ad. 7 genetic materials in Ad. $2^{+(t7)}$ and Ad. $12^{+(t7)}$ are in the same capsid.

A third type of experiment, utilizing the transfer procedure of Rapp *et al.*,[6] provided direct evidence on this point. Dilutions of Ad. $2^{+(t7)}$ were used to infect plaque dishes of AGMK cells simultaneously seeded with nonhybrid Ad. 12 at a multiplicity of 0.5 HEK plaque-forming units per cell. The Ad. 12 alone does not form plaques under these conditions; at the dilutions employed, plaques form chiefly where Ad. 2^{+} hybrid particles infect cells also hit with Ad. 12, and the progeny are Ad. 12^{+}.[6] Seven plaque isolates were obtained from these dishes by passage to AGMK cells; all seven induced both SV40 and Ad. 7 T antigen when inoculated into HEK cells, and the induction of both antigens was prevented by mixture with Ad. 12 antiserum but not by Ad. 2 antiserum. Thus, in transfer experiments in which a single Ad. $2^{+(t7)}$ hybrid particle was the donor, both the SV40 and Ad. 7 genetic materials went together, leaving no alternative but that they are in the same capsid.

Discussion.—The results presented here establish that two adenoviruses to which the SV40 gene was transferred from Ad. 7 strain $E46^{+}$ have also acquired a portion of Ad. 7 genetic material. The consistency with which the two antigenic determinants were transferred together from $E46^{+}$, the inability to separate them by cloning, and the relatively consistent ratios of staining percentages with SV40- and Ad. 7-tumored hamster sera strongly indicate linkage between the two genetic materials. An alternative hypothesis which cannot be ruled out is that in the formation of the defective particles a number of short DNA segments are encapsidated, so that by chance each particle would have determinants of both types. Biophysical studies of the viral DNA may answer this question.

The possibility that the SV40 DNA activates a gene of Ad. 2 to produce an antigen cross-reactive with Ad. 7 was ruled out by the negative results with Ad. 2-SV40 hybrids obtained from SV40 or Ad. 1^{+}.

It seems most unlikely that Ad. $2^{+(t7)}$ and Ad. $12^{+(t7)}$ have acquired the entire Ad. 7 genome. There is no evidence of formation of Ad. 7 capsid proteins (as judged by the failure of Ad. 7 rabbit antiserum to neutralize) or, in the case of Ad. $2^{+(t7)}$, of that Ad. 7 T antigen which is stained in FA tests by Ad. 12-tumored hamster sera which do not react with the parent Ad. 2 prototype. There is suggestive evidence, however, that the hybrid particles contain an amount of DNA comparable to that of the complete adenovirus genome, in that equilibrium density gradient centrifugation gave no separation of the complete and defective particles.[8]

The antigens produced during infection with the various adenoviruses constitute a highly complex group. The antigens can be classified by a number of parameters:

early antigens not inhibited by FUDR or late antigens which are inhibited by FUDR; antigens present in standard virus harvests, early cell pack preparations, and/or in tumors; those reactive in CF, FA, and/or immunodiffusion tests; group-reactive, subgroup-reactive, or type-specific; heat-stable or heat-labile; structural or nonstructural; and of various morphologic appearances in FA tests. The relationships between the antigens classified in these various ways are far from established, so that at present many of the antigens can be identified only in relation to one assay system. The Ad. 7 antigen induced by the transfer hybrids is of the early, FUDR-resistant type; is present in Ad. 7 tumors, but possibly as a minor antigen, since the Ad. 7-tumored hamster serum used for its detection does not stain Ad. 7 tumors; and is probably subgroup-reactive or type-specific. It is possible that this is the same subgroup-reactive antigen detected in Ad. 7 cell pack antigens by CF testing with Ad. 3- and Ad. 7-tumored hamster sera.[7]

It does not appear that the SV40 genetic material in E46$^+$ was necessary for the transfer of the Ad. 7 gene to Ad. 2 capsids. Preliminary studies[14] suggest that mixed infection of HEK cells with nonhybrid Ad. 2 and Ad. 7 strains does result in some transfer of an Ad. 7 antigenic determinant. However, without a selective growth advantage such as that afforded to adenovirus-SV40 hybrids by the SV40 genetic material, it has not been possible to obtain the transfer particles in high titer or maintain them in serial passage for study.

Summary.—Strains of Ad. 2 and Ad. 12 to which the SV40 gene for T antigen induction was transferred by mixed infection with the Ad. 7-SV40 hybrid strain E46$^+$ also acquired the ability to induce a T antigen of Ad. 7. The determinant for induction of the Ad. 7 antigen is in Ad. 2 or Ad. 12 capsids, is not separable from the SV40 gene by cloning, and like the SV40 DNA, is in defective particles. It is concluded that the SV40 DNA and a portion of Ad. 7 DNA are in the same capsids, and are probably linked.

We are deeply indebted to Dr. Robert J. Huebner for supplying the adenovirus-tumored hamster sera used in these studies.

* This work was partially supported by the National Cancer Institute Field Studies.

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