IN VITRO TRANSFORMATION BY THE ADENOVIRUS-SV40 HYBRID VIRUSES

II. Characteristics of the Transformation of Hamster Cells by the Adeno 2–, Adeno 3–, and Adeno 12–SV40 Viruses*

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The adenovirus (adeno)-SV40 hybrid viruses offer a unique opportunity to study the phenomenon of transformation induced by both the adenoviruses and SV40. In a previous report, transformation of hamster and human tissue culture cells with the adeno 7–SV40 hybrid virus was described (1). Although the transformation which resulted was characteristic of SV40, there was some evidence, from serologic studies, that a portion of the adenovirus genome was operative in the transformed cell as well (2).

More recently, other adeno-SV40 hybrids have been recovered from various passage lines used for vaccine production (3) or have been formed with both the nononcogenic and oncogenic adenoviruses by transcapsidation procedures (4, 5). The existence of these hybrid viruses has raised questions regarding the amount and physical state of the SV40 DNA contained within the hybrid particle. Rowe and Pugh (6) and Baum et al. (7) have shown that the SV40 and adeno 7 DNA's are physically linked in the adeno 7–SV40 hybrid virus, and that a portion of the adeno 7 DNA accompanies the SV40 DNA when the latter genome is transferred from the adeno 7–SV40 hybrid to another adenovirus (6). Presumably, such linkage occurs with the nononcogenic adeno-SV40 hybrid viruses as well. If transformation could be effected with the various hybrid viruses one might be able to determine whether nononcogenic adenovirus DNA could be integrated in a host cell genome and produce a T antigen as occurs with the oncogenic adenoviruses (8). In addition, an examination of the determinants of the resulting transformation, whether derived from the SV40 or adeno genomes or both, might give information on variable phenotypic expressions of the separate viral genomes on a host cell. It would also be of interest to determine

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the oncogenic potential of the adeno 7–SV40 hybrid, and any markers of the portion of the adeno 7 DNA present, when this hybrid is enclosed in the protein coat of an oncogenic or nononcogenic adenovirus as occurs in the transfer hybrids.

This communication describes the transformation of hamster kidney tissue culture cells by the adeno 2–SV40, adeno 3–SV40, and adeno 12–SV40 hybrid viruses, by adenovirus type 12, and a mixture of adenovirus type 12 and

TABLE I

Summary of the Adenovirus-SV40 Hybrid Preparations Used in Transformation Studies

Hybrid preparation	Strain	Particles in preparation	Reference
Passage			
Ad 2^{++}	Ind. 2	Adeno 2 "Hybrid"* particle SV40	12
Ad 3^{+}	JF	Adeno 3 Adeno 3–SV40	3
Ad 7^{+}	LLE46	Adeno 7 Adeno 7–SV40	10
Transfer			
Ad 2^{+t7}	Ad-6	Adeno 2 Adeno‡-SV40	4
Ad 12^{+t7}	Huie	Adeno 12 Adeno§–SV40	4

* See text.

‡ It has not been established whether the hybrid particle, in addition to the adeno 7 genome, contains the adeno 2 genome.

§ It has not been established whether the hybrid particle, in addition to the adeno 7 genome, contains the adeno 12 genome.

SV40, and the characteristics of the transformed cell lines which were established. In the accompanying paper, a morphologic description of the tumors induced by transplantation of the transformed cells to hamsters will be presented (9).

Materials and Methods

Virus.—In table I, a summary of the various adeno-SV40 hybrid viruses used in the transformation studies is given. Two classes of hybrid preparations were employed. The first type originated by passage of various adenovirus pools in rhesus monkey kidney cultures which contained SV40 (3) and has been thoroughly described for the adeno 7–SV40 hybrid virus (10). The adeno 3 (strain JF)–SV40 hybrid, hereafter referred to as Ad. 3^{+}, was produced in this

way (3). The pool used in the present experiments was derived from the third primary African green monkey kidney (AGMK) passage. As with the adeno 7–SV40 hybrid preparation (Ad. 7^+), this hybrid population contains two particles: the hybrid particle, composed of portions of the adenovirus and SV40 genomes, and complete adenovirus virions. The nonhybrid adenovirus 3 used in the present experiments was isolated from the hybrid preparation by the limiting dilution technique (11).

The Ind. 2 hybrid strain of adenovirus 2 was produced in a similar way and has been described in detail (12). The pool used in these studies consisted of virus banded in a RbCl preparative density gradient and was supplied by Dr. A. Lewis, Jr. This population contains at least three particles: complete adeno 2 virions ($10^{9.5}$ $TCID_{50}$/ml); "hybrid"[1] particles which yield SV40 virus ($10^{8.8}$ $TCID_{50}$/ml); and a small number of free SV40 particles (10^4 $TCID_{50}$/ml). The infectivity titers were determined in human embryonic kidney (HEK) (adeno virions) and AGMK (hybrids and SV40) cultures. This hybrid preparation will be referred to as Ad. 2^{++}.

The second class of hybrids are the transcapsidants. These hybrid viruses (adeno 2^{+t7} and 12^{+t7}) were produced by growing the Ad. 7^+ hybrid preparations with adenoviruses types 2 and 12 respectively and then adding adeno 7 antiserum. In this way, the portion of the SV40 genome contained within the adeno 7 hybrid particle, together with some adeno 7 DNA, were transferred to adenoviruses 2 or 12 (4, 5).

The adenoviruses types 2 (strain Ad-6) and 12 (strain Huie) used in the present experiments were from the first and second HEK passages respectively, having been previously passed in KB or HeLa cell cultures.

The origin and history of SV40, strain 777, ($LLCMK_2$, $AGMK_5$, $BSC\text{-}1_2$) have been described in detail (13). The virus pool used in these experiments was derived from the third passage in the continuous cell line, BSC-1 (14) derived from primary AGMK cells.

Cell Cultures.—HEK and AGMK roller tube cultures were obtained from Flow Laboratories, Rockville, Md., and Microbiological Associates, Inc., Bethesda, Md., respectively. They were maintained with Eagle's basal medium which contained 2% heated (56°C, 30 min) agammaglobulinic calf serum, 2 mM glutamine, and penicillin and streptomycin in concentrations of 100 units and 100 μg per ml respectively.

Primary weanling hamster kidney (WHK) monolayer cultures in roller tubes were obtained from Microbiological Associates or Flow Laboratories. They were maintained in NCTC 109 medium with 10% heated (56°C, 30 min) fetal calf serum, 2 mM glutamine, and antibiotics. Some experiments were carried out in the presence of 1.8 or 0.1 mM calcium. The media for these experiments consisted of Eagle's minimum essential medium without calcium, 5% unheated, dialyzed calf serum, 2% unheated fetal calf serum, non-essential amino acids, 2 mM glutamine, and antibiotics. Calcium chloride was added to a final concentration of either 1.8 or 0.1 mM. All cultures were maintained in a stationary position at 37°C and the medium was changed twice weekly.

WHK cultures were inoculated with 0.1 ml of the various hybrid, adeno or SV40 virus preparations (see Tables II and III) and observed 2–3 times each week. When transformation was well advanced, the cells were dispersed with trypsin and passed. The cell lines thus derived were maintained in the NCTC 109 medium described above; a portion of the cells were frozen while the remainder were passed at approximately weekly intervals.

All negative cultures were observed for at least 70 days and some for as long as 133 days.

Virus Isolation Tests.—The presence of SV40 and adenovirus in the transformed cell lines was tested for by inoculation of supernatant fluid, extracts of 10% cell suspensions, and various

[1] Those strains in which linkage has not been definitely established will be referred to as "hybrid" populations while the adeno 3– and adeno 7–SV40 hybrids and the transcapsidants derived from the latter hybrid can be referred to as true hybrids.

concentrations of viable cells into AGMK and HEK cultures. All cultures were examined 2–3 times each week for the appearance of SV40 or adenovirus cytopathogenic effect (CPE); the cultures inoculated with supernatant fluid and cell extracts were observed for 4–6 wk, while the cultures overlaid with viable cells were observed for 2–3 wk. All negative cultures were frozen and thawed two to three times, the replicate tubes pooled, and the contents blind passed to fresh cultures which were observed for 4–6 wk. The details of these procedures have been described elsewhere (13, 15).

Serological Studies.—The antigen content of the transformed cell lines was examined by complement fixation (CF) and fluorescent antibody (FA) procedures which have been described in detail elsewhere (16, 17). For CF tests, the microtechnique was used, employing 1.6–1.8 exact units of complement. SV40 and adeno T antigens were tested for with 4–8 units of antibody from hamsters with SV40 and the various adenovirus tumors respectively. 4–8 units of SV40 viral or pooled, convalescent, human adenovirus sera were employed to detect SV40 and adenovirus antigens respectively. The preparation of 10% cell pack antigens, usually at 24 hr after the infection of HEK cultures with the various adenoviruses, has been described in detail (18). Some of these HEK-infected cultures were maintained in the presence of 10^{-5} M 5-fluorodeoxyuridine (FUDR) to block DNA synthesis and viral replication and thereby obtain cells containing the early antigens coded for by input viral genome.

FA tests were done with cells grown on cover slips as previously described (17). For detection of SV40 and adeno T antigens the indirect FA test was employed, utilizing sera from hamsters bearing SV40 and the various adenovirus-induced tumors and fluorescein-conjugated goat anti-hamster serum. For detection of SV40 viral antigen the direct FA test was used, employing a fluorescein-conjugated African green monkey hyperimmune anti-SV40 serum.

Transplantation to Hamsters.—The transformed cell lines at various passage levels were transplanted to hamsters (see Table I, reference 9). Portions of the tumors that arose were made into 10% suspensions which were analyzed for virus and antigen content by methods previously described (13).

Chromosome Studies.—Cells from transformed cultures were planted in 60 mm plastic Petri dishes. When the cells were subconfluent the medium was changed and the cultures reincubated in 5% CO_2 for 18 hr. Colcemide (Ciba Pharmaceutical Co., Summit, N. J.) was added (final concentration 0.1 μg per ml) and the cultures reincubated for 3 hr at 37°C. The cells were loosened with 2 ml of 0.1% trypsin in phosphate-buffered saline (PBS), washed with 5 ml of PBS, treated with 1% sodium citrate for 2–10 min, and fixed with fresh acetic methanol (1:3) for 10 min. After resuspension in 0.5 ml of fresh fixative, several drops of the suspension were placed on cover slips and allowed to dry on a slide warmer at 40°C. Cover slips were stained with lactic-acetic-orcein and examined with the phase microscope. Uninfected control cultures were processed for chromosome study in the same manner as the transformed cultures. However, chromosome analysis of control cultures was restricted to the first passage because of progressive failure of growth and mitosis during subsequent passages.

Chromosome counts were done on well spread metaphase figures. In addition, each cell was scored for the number of chromosomes in groups 16–19 (medium sized acrocentric), 20 (small metacentric), 21 (small acrocentric), and for the presence of the X chromosome (largest metacentric). Each figure was also examined for marker chromosomes, dicentrics, rings, minutes, fragments, chromatid and isochromatid breaks or gaps, and other aberrant chromosomes. 100 cells were examined in each preparation; the cultures were examined without knowledge of the virus involved.

RESULTS

Transformation of WHK Monolayer Cultures by the Various Hybrid Viruses.—In Tables II and III the results of transformation experiments carried out with

TABLE II

Summary of WHK Tran.formation Experiments with Adeno 2 and Adeno 3 Hybrid Viruses

Virus	Dose/tube	Maximum CPE	Frequency of transformation, *No. tubes pos./ No. tubes inoc.*	Day transformation first observed	"Pattern" of transformation
	Log_{10} $TCID_{50}$*				
Ad. 2^{++}‡	7.5	+++-++++	3/10	20	SV40
" 2^{+t7}	5.5	+-++	5/5	14	SV40
	4.5	±	3/5	38	SV40
" 2	7.2	++++	0/5	—	
	6.2	++++	0/5	—	
	5.2	+	0/5	—	
" 3^{+}	6.5	+-++	5/5	17	SV40
" 3	6.7	+-++	0/5	—	

* The infectivity titrations of the hybrid viruses listed here and in table III were carried out in HEK cultures and represent the titer of infectious adenovirus particles. The titer of the hybrid particles is approximately the same, as determined by SV40 T antigen induction in HEK cultures (33).

‡ Virus was exposed to approximately 50 units of SV40 antiserum at room temperature for 45 min; maintenance media contained antiserum.

TABLE III

Summary of WHK Transformation Experiments with Adeno 12^{+t7}, Adeno 12, and Adeno 12 + SV40 Viruses

Virus	Dose/tube	Maximum CPE	Frequency of transformation, *No. tubes pos./No. tubes inoc.*	Day transformation first observed	Avg. No. foci/ tube*	"Pattern" of transformation
	Log_{10} $TCID_{50}$					
Ad. 12^{+t7}	6.5–6.8	+-++	16/17	17–32	2	Adeno (SV40)
" 12	6.5	±-+	5/15	24–109	1	Adeno
" 12 + SV40	6.5/8.5	±-+	7/7	12–14	21	Adeno
SV40	8.5	0	7/8	21–38	3–4	SV40

* Average number of foci per transformed culture.

the various hybrid viruses are given. After inoculation of WHK cultures with the hybrid or adenoviruses, there was a variable CPE characteristic of the adenoviruses which, for the most part, regressed after involvement of 25–50% (1+–2+) of the cell sheet. Generally, one to three foci of transformed cells

appeared in cultures transformed by the Ad. 2^{+t7}, Ad. 2^{++}, and Ad. 3^{+} hybrids. This was usually preceded by the appearance of multinucleated, syncytial giant cells. The transformed cells were of a triangular to polygonal shaped "epithelioid" morphology which resembled the transformations previously described for SV40 and the Ad. 7^{+} viruses (15, 1). Transformation was never observed in cultures inoculated with adenoviruses types 2 or 3.

Transformation with Ad. 12^{+t7} occurred both earlier and with a greater frequency than with adeno 12 (Table III). Since the SV40 genome, or part thereof contained within the Ad. 12^{+t7} hybrid, presumably enhanced the transformation potential of adeno 12 (*vide infra*), cultures were inoculated with a mixture of SV40 and adeno 12 viruses. This resulted in the earliest appearance of transformation; moreover, up to 35 transformed foci appeared in these cultures when counts were made 2 wk after the recognition of the first transformed foci. Only 1–2 transformed areas appeared in comparably handled Ad. 12^{+t7}– or adeno 12–infected cultures. It is unlikely that this large number of foci, at the time the counts were determined, was due to seeding from older foci since dissemination of transformed cells did not occur in tubes containing only one focus for 2–4 wk after the transformation was first observed; after this period secondary colony formation did occur. The earlier and more extensive transformation by both Ad. 12^{+t7} and the adeno 12–SV40 mixture when compared to adeno 12 alone occurred in media which contained either 1.8 or 0.1 mM Ca. These experiments were undertaken since it has been demonstrated that transformation of rat embryo cells by adeno type 12 occurs more reproducibly and to a greater extent in media containing low concentrations of calcium (19).

The transformed foci appearing after inoculation of adenovirus type 12 were composed of small, tightly packed epithelial cells with dense, multilayered central portions which often retracted after 2–3 wk. In general, the morphology of transformed cells produced by the Ad. 12^{+t7} hybrid or by inoculation of a mixture of adenovirus type 12 and SV40 was similar to the morphology of adeno 12–transformed cells; however, foci resembling those produced by SV40 were occasionally observed in cultures transformed by the Ad. 12^{+t7} hybrid.

Characteristics of Cell Lines

Origin.—When the transformed cells had grown to involve approximately half of the glass surface of the tube bathed by medium, the cells were dispersed with trypsin and passed. The following cell lines were thus derived from hybrid-transformed cultures: four lines from Ad. 2^{+t7}-transformed cultures; two lines each from Ad. 2^{++}- and Ad. 3^{+}-transformed cultures; and one line each from cultures transformed with Ad. 12^{+t7} and adeno 12 viruses (see Table IV).

Morphology.—Cover slip preparations containing confluent monolayers or individual colonies of the cell lines at the fourth to seventh passage levels were fixed in 10% buffered formalin and stained with hematoxylin and eosin. The

TABLE IV

Virus and Antigen Content of Transformed Cell Lines and Tumors

Transformed cell line	Cell line					Tumor						
		Virus		T antigen			Virus		T antigen*		T antibody*	
	Passage no.‡	AGMK	HEK	SV40	Adeno	Passage no.‡	AGMK	HEK	SV40	Adeno	SV40	Adeno
Ad. 7^+ HK-1§	2	0	0	+	0	6	0/1	0/1				
" 2§	—	—	—	+	0	4	0/7	0/7	9/22‖	0/22	14/28¶	4/28**
						11	0/15	0/15				
" 3‡‡	15	0	0	+	0	17	0/5	0/5				
Ad. 2^{+t7} HK-1	7	0	0	+	0	3	0/6	0/6	12/12	0/12	11/14	1/14**
" 2	7	0	0	+	0	3	0/8	0/8				
" 3	5	0	0	+	0		—	—				
" 4	4	0	0	+	0		—	—				
Ad. 2^{++} HK-1	8	+ (SV40)	0	+	0	5	0/5	0/5				
" 2	8	+ (SV40)	0	+	0	5	0/5	0/5	4/4	0/4	10/10	1/10**
Ad. 3^+ HK-1	8	0	0	+	0	5	0/1	0/1	2/2	0/2	2/2	0/2
" 2	8	0	0	+	0	5	0/1	0/1				
Ad. 12^{+t7} HK-1	5	0	0	+	+	3	0/8	0/8	8/8	8/8	0/8	8/8
Ad. 12 HK-1	6	0	0	0	+	4	0/8	0/8	0/8	8/8	0/8	8/8

For adeno T antigens, Ad. 7^+, 2^{+t7}, 2^{++}, and 3^+ HK cells were tested by FA and transformed and tumor cell extracts by CF with sera from hamsters bearing tumors induced with adenoviruses 3, 7, and 12.

Ad. 12^{+t7} and 12 HK cells and tumors were tested for adeno 12 T antigen with sera from hamsters bearing adeno 12 tumors.

For adeno T antibody in hamsters bearing tumors induced with the Ad. 7^+, 2^{+t7}, and 3^+ HK lines, antigens prepared from hamster tumors induced with adenoviruses 3, 7, and 12 and from HEK cells infected with adenoviruses 2 (with and without FUDR), 3, 7, and 12 were used.

Adeno 12 T antibody was tested with antigens prepared from adeno 12 tumors.

* The results of antigen and antibody determinations were pooled when more than one cell line transformed by any hybrid preparation was transplanted to hamsters.

‡ Passage level at which either virus and antigen content determined or cells were inoculated into hamsters.

§ These cell lines were formerly designated $E46^+$ HK 1–2 (see reference 1).

‖ No. tumors positive/no. tumors tested which were not anticomplementary.

¶ No sera positive/no. sera tested.

** These sera were positive when tested with two adeno 7 hamster tumor extracts.

‡‡ Ad. 7^+HK-3 and 4 (see Fig. 1 and Table V) were established at a later time; the properties of these cell lines do not differ from those described for Ad. 7^+ HK 1–2.

Ad. 7^{+}–, Ad. 2^{+t7}–, Ad. 2^{++}–, and Ad. 3^{+}–transformed lines were morphologically similar. The colonies were round and were composed of epithelioid to triangular shaped cells which had a slight tendency to pile up in the center. The nuclei were large, pleomorphic, round to ovoid, and contained approximately 6–10 nucleoli; in addition, the nuclei contained coarse masses of irregularly shaped chromatin. The nuclear: cytoplasmic ratio was increased. Many multinucleated giant cells were present, some containing up to 16 central nuclei. Eosinophilic cytoplasmic inclusions were rarely observed. All of these characteristics have been observed with WHK cells transformed by SV40 (20). In addition, some colonies, derived predominantly from the Ad. 2^{+t7} HK lines, were composed of large anaplastic cells which contained large round nuclei with prominent nucleoli which filled most of the cell.

The adeno 12 HK-1 cell line gave rise to round to irregular shaped colonies composed of small, epithelial cells with dense, multilayering of the cells in the central portions of the colony. Large, pleomorphic, round or irregular shaped nuclei filled practically the entire cell. Scant basophilic cytoplasm was present; however, the nuclei were smaller than the SV40 type nucleus described above. The nuclear chromatin pattern was fine and granular and the nuclei appeared pale, often containing one to three small nucleoli per cell.

Colonies derived from the Ad. 12^{+t7} HK-1 cell line resembled those produced from the adeno 12 HK-1–transformed line; the cellular morphology was similar as well. However the nuclei frequently contained two to five prominent nucleoli with many coarse clumped chromatin masses and small particulate chromatin grains. Giant cells and occasional eosinophilic cytoplasmic inclusions were also present.

Thus, the Ad. 7^{+}–, Ad. 2^{+t7}–, Ad. 2^{++}–, and Ad. 3^{+}–transformed lines contained cells and produced colonies which were alike and similar to SV40-transformed WHK cells, except for the anaplastic variant described in the Ad. 2^{+t7} lines. The adeno 12 HK line was distinct with respect to cell and colonial morphology. The Ad. 12^{+t7}–transformed line resembled the latter line but the cells had nuclei with characteristics of both SV40 and adeno-transformed WHK cells, and contained giant cells and eosinophilic inclusions as well.

Virus Isolation.—The results of virus isolation studies are given in Table IV. Supernatant fluids and extracts of concentrated cell pack preparations of the transformed cell lines, at the passage levels indicated in Table IV, were all negative for virus when inoculated into both AGMK and HEK cultures and blind passed. Infectious SV40 was isolated from both Ad. 2^{++}–transformed lines by blind passage of the overlaid AGMK cultures and from 2 of the 10 tumors induced by transplantation of these transformed cells. No infectious virus was isolated from tumor suspensions prepared from tumors induced with the other hybrid or adeno 12–induced transformed cell lines when the suspensions were inoculated into AGMK and HEK cultures which were subsequently blind passed.

Serologic Studies

The results of serological studies done on the transformed lines and tumors induced by transplantation of these cells to hamsters are given in Table IV. SV40 T antigen was demonstrated by both CF and FA tests in all hybrid-transformed cell lines and in most tumor extracts by CF. Virtually every cell contained the SV40 T antigen when these were examined by FA tests. In order to determine whether any of the transformed colonies might not contain the SV40 T antigen, cover slips were seeded with small numbers of cells from three Ad. 2^{+t7} HK and from the Ad. 12^{+t7} HK cell lines. When colonies containing more than approximately 64 cells had formed, the cover slips were fixed and stained for SV40 T antigen by indirect FA tests. Of 41 and 50 colonies from the Ad. 2^{+t7} HK and the Ad. 12^{+t7} HK lines respectively, all contained nearly 100% positive cells. Thus the SV40 genome is present in probably all the cells transformed by at least these two hybrid viruses. The antigen titers of extracts of the hybrid-transformed cell lines and tumors were unusually high, the average titer being 1:16–1:32. The highest titers were present in tumors induced with Ad. 12^{+t7}–transformed cells; of eight tumors, 32 units of antigen were present in two tumors, 64 units in four tumors, while two tumors contained 128 units of antigen. No SV40 viral antigen was ever detected in transformed cell or tumor extracts.

Adeno 12 T antigen was present in Ad. 12^{+t7} and adeno 12–transformed cells as determined by nuclear fluorescence and tumor and transformed cell extracts when tested by CF, but T antigens could not be demonstrated in cells transformed by the other hybrid viruses with the materials enumerated in Table IV. The inability to detect adeno T antigens in CF tests, especially in Ad. 7^+ and 3^+ hybrid–transformed cells with serum from hamsters bearing tumors induced with adenoviruses 7 and 3 respectively, may not be due to the absence of these antigens; tumors induced with the Ad. 7^+ hybrid-transformed cells had no detectable adeno 7 T antigen, yet 4 of 28 hamsters developed specific antibody to this antigen.

Most of the animals bearing tumors induced with hybrid-transformed cells developed SV40 T antibody. The inability to detect this antibody in hamsters bearing Ad. 12^{+t7} tumors, particularly since these tumors had the highest levels of SV40 T antigen, is not clear at present. All of these animals developed antibody titers of from 1:40–1:320 against the adeno 12 tumor antigen.

Two sera from hamsters bearing tumors induced with one each of the Ad. 2^{+t7} HK and Ad. 2^{++} HK cell lines reacted in CF against two adeno 7 tumor extracts at dilutions of 1:80 or 1:160. However, approximately half of the sera from animals bearing tumors induced with Ad. 2^{+t7} and Ad. 2^{++} HK cells stained HEK cells, in indirect FA tests, infected with adenoviruses 7 and/or 2 in the presence of 10^{-5} M FUDR. The fluorescence was confined to the nucleus and was characteristic of that described for the adenovirus T antigens which are present in tumor cells induced with the oncogenic adenoviruses (32). The

sera did not stain cover slips which had been heated at 56°C for 30 min. Thus, the morphology, FUDR resistance, and heat lability of some of the antigens stained by the sera from animals bearing tumors induced with Ad. 2^{+t7} and Ad. 2^{++} HK cells suggest that these sera contain antibody(s) to the adenovirus-specific T antigens. More sera from hamsters with Ad. 2^{+t7} HK cell-induced tumors stained adeno 7 than adeno 2–infected, FUDR-treated HEK cells, while the reverse was true with sera from hamsters with Ad. 2^{++} HK cell–induced tumors; these reactions suggest that the adenoantigens present in Ad. 2^{+t7} and Ad. 2^{++} HK cells are coded for by the adeno 7 and 2 genomes respectively. It is not yet known, however, to what extent the T antigens of these two viruses cross-react or whether any adeno 2 genetic material is present in the Ad. 2^{+t7} HK cells. Most of the sera from these tumor-bearing animals also stained HEK cells infected with adenoviruses 2 and/or 7 in the absence of FUDR. This staining was more characteristic of adeno "viral" nuclear fluorescence. Moreover, ascites fluids prepared by repeated intraperitoneal inoculation of hamsters (31) with tumors derived from Ad. 2^{+t7}– or Ad. 2^{++}–transformed cells stained HEK cover slips infected with adenoviruses 2, 7, and 12. The presence of cross-reactions with the ascites fluids suggests that adenovirus antigen(s) shared by several adenoviruses are present in these transformed cells. These latter antigens may be viral in nature since the ascites fluids do not stain HEK cells infected in the presence of FUDR. Further studies concerning these preliminary staining reactions are in progress. Since the majority of the reactions detected by fluorescence microscopy were negative in CF tests, it would seem that the former is a more sensitive procedure for detecting both early and late adenovirus antigens than is CF testing.

No adenovirus 2–neutralizing antibody was detected in any of the sera from hamsters bearing tumors induced with Ad. 2^{+t7}– and Ad. 2^{++}–transformed cells when tested at a 1:2 dilution against 1000 $TCID_{50}$ of adenovirus 2.

From the serologic studies, one may conclude that at least a portion of the SV40 genome is permanently associated with the genome of all the hybrid-transformed cells, as evidenced by T antigen formation. With Ad. 12^{+t7}–transformed cells, this is true of the adeno genome as well. Some evidence that the adenovirus genome is also present in the Ad. 7^{+} HK tumor cells is gained from the serologic data. There is also evidence that portions of the adeno 7 and 2 genomes are present in Ad. 2^{+t7} and Ad. 2^{++} HK cells respectively which code for early and late adenovirus antigens.

Cytogenetic Studies

Chromosome Number.—Chromosome number distributions of cell lines derived from cultures transformed by SV40, adeno 12, and the Ad. 12^{+t7}, 7^{+}, and 2^{+t7} viruses are shown in Fig. 1. Chromosome number distributions of SV40– and Ad. 12^{+t7}–transformed lines were similar. Both exhibited marked hetero-

ploidy with counts ranging from hypodiploid to hypertetraploid. Heteroploidy was less extensive in cells transformed by adenovirus 12 and the Ad. 7^+ and 2^{+t7} hybrids. In the adenovirus 12 HK-1 line, a clear modal number of 43 was established, although 24% of the cells were in the hyperdiploid to near tetraploid range. Chromosome number was distributed in the hyperdiploid to hypotetraploid range in the Ad. 7^+ HK-4 cell line; 93% of the counts were in the 50–70 range at the fourth passage. At the eighth passage, the chromosome number

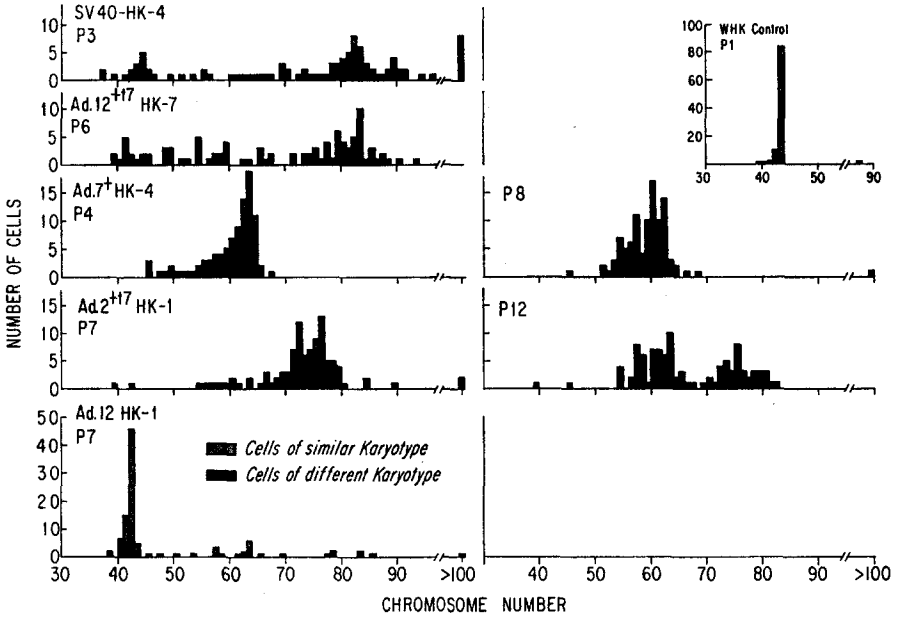

FIG. 1. Distributions of chromosome numbers of WHK cell lines transformed by Adeno 12, hybrid viruses Ad. 2^{+t7}, 7^+, and 12^{+t7}, and SV40. Passage numbers are given, and cells of the same chromosome number with the same number of chromosomes in groups 16–29, 20, 21, and X are indicated as similar in karyotype.

distribution was very similar and 98% of cells were in the 50–70 range. Chromosome number was predominantly in the subtetraploid range in the Ad. 2^{+t7} HK-1 cells at the seventh passage, with 74% of counts between 70 and 80. By the 12th passage heteroploidy was increased and 98% of counts ranged between 50 and 85 with a modal number of 64.

The modal number of uninfected WHK cells after the 1st passage was the normal diploid number, 44. One tetraploid cell was noted, and another 15% of the cells were near diploid (40, 41, 42, and 43). While some WHK cultures were maintained for three serial passages, the rate of mitosis was not sufficient for counting and karyotype analysis after the second passage.

Chromosome Constitution.—The results of microscopic analysis to determine the identity of certain chromosomes in cells with the same chromosome number are indicated in Fig. 1. The number of chromosomes found in groups 16–19, 20, 21, and X was utilized as an index of similarity, but not necessarily identity, between such cells. Cells with the same number of chromosomes in all of these groups were scored as "similar" in karyotype. No instances of similarity between cells with the same chromosome number were found in the Ad. 12^{+t7}–transformed cell population; two groups of two cells with similar karyotypes were noted in the SV40 cells. The greatest degree of similarity was present in the adeno 12 material. Of 46 cells with the modal number of 43, 39 were found to be similar. These cells had the normal number of chromosomes in the group scored and one X chromosome, and further karyotyping is in progress to determine if all have identical karyotypes. However, the similarity of karyotype indicates considerably greater stability than the SV40 and adenovirus-SV40 hybrid cell populations.

The majority of cells in the Ad. 7^{+} material with the same chromosome number were dissimilar at both the fourth and eighth passages (see Fig. 1). Cells with unstable karyotype predominated in the Ad. 2^{+t7} culture at the 7th passage and were present exclusively at the 12th passage, when no instances of karyotype similarity were noted. Karyotype was therefore most unstable in the cultures with extensive heteroploidy (SV40, Ad. 12^{+t7}, and Ad. 2^{+t7} passage 12 transformed cells), of low stability in the adenovirus 7^{+} and 2^{+t7} passage 7 cells, and most stable in adenovirus 12–transformed cells.

All of the modal number, 44, cells in the control preparation had a normal number of chromosomes in the groups utilized to determine karyotype similarity, indicating a stable diploid karyotype. Loss of chromosomes from both these and other groups accounted for the hypodiploid population (16%), although loss of a chromosome from groups other than those scored was most frequent.

Chromosomal Aberrations.—The percentage of cells in the transformed populations with certain abnormalities is illustrated in Table V. Two marker chromosomes, a long subtelocentric and a long dicentric, were found in a significant percentage of the SV40 cells. At least one long dicentric was observed in all of the other infected populations including adeno 12–transformed cells (3%), indicating it is not a unique effect caused by the SV40 genome. Other dicentric chromosomes of varying sizes, found in 49% of the SV40 cells, were present in all of the other infected cultures to a lesser but significant degree. The long subtelocentric marker found in 16% of the SV40 and 7% of the Ad. 12^{+t7}–transformed cells was observed in 1% of the adeno 12–transformed cells, indicating it also is not unique to SV40. It is noteworthy that gaps and breaks were most frequent in cells transformed by adeno 12, although these did not demonstrate marked damage, ring and multicentric chromosomes, or elimination of X chromosome(s).

Marked damage (see Table V) was evident in a significant percentage of SV40-transformed cells at the 3rd passage and Ad. 2^{+t7} cells at the 12th passage. Ring and multicentric chromosomes and minutes were most frequent in the SV40 material. An interesting finding was the high percentage (67 at passage 7 and 38 at passage 12) of cells in the Ad. 2^{+t7}–transformed cell population without any chromosome having the morphology typical of the X chromosome. The X chromosome(s) was also apparently missing in the two near diploid cells counted. Further investigations to determine if there has been a partial

TABLE V

Percentage of Cells with Aberrant Chromosomes and other Abnormalities

Cell line	Passage No.	Long subtelocentric marker	Total dicentrics / Long dicentric marker	Ring chromosomes	Multicentric chromosomes	Other atypical chromosomes	Absence of X chromosomes	Fragments	Minutes (1 or more per cell)	Chromatid and isochromatid gaps and breaks	Marked damage to cell*
SV40–HK-4	3	12	39/7	10	2	25	9	17	34	11	9
Ad 7^{+} HK-4	4	0	9/1	2	0	20	11	15	15	3	1
Ad. 7^{+} HK-4	8	0	6/0	1	0	26	1	16	25	8	0
Ad. 12^{+t7} HK-1	6	7	3/1	1	1	22	4	3	7	11	1
Ad. 2^{+t7} HK-1	7	0	10/1	1	0	14	67	11	19	6	0
Ad. 2^{+t7} HK-1	12	2	21/1	1	0	45	38	16	26	11	4
Ad. 12 HK-1	7	1	4/3	0	0	4	0	3	8	27	0
WHK control	1	0	0/0	0	0	1	0	0	0	5	0

* More than four aberrant chromosomes or abnormal events per cell. Each chromatid and isochromatid break or gap and each isolated fragment not related to any adjacent broken chromosome were scored as one abnormal event. 1 or more minutes in a cell scored as one abnormal event.

deletion, or other structural rearrangement of the X chromosome are now in progress.

Only one obvious chromosomal aberration was noted in the control preparation (a long acrocentric in one cell) and 5% of the cells had chromatid and isochromatid gaps or breaks. No other aberrations were noted in the control cells.

In summary, cells with extensive damage and aberrant chromosomes were more frequent in the extensively heteroploid cell population transformed by SV40 and in the Ad. 2^{+t7} cell population after 12 serial passages. Cells transformed by adenovirus 12 were the most stable with respect to chromosome number and similarity of karyotype, a characteristic distinguishing them from cells transformed by the hybrids and SV40. It was not possible to distinguish cell populations transformed by the hybrids on the basis of chromosome number distribution, markers, atypical chromosomes, or other aberrations, and all three hybrid-transformed lines examined exhibited characteristics typical of SV40.

Also, the cell lines derived from transformations by adenovirus 12 and the hybrids 2^{+t7}, 7^{+} and 12^{+t7} could not be distinguished from SV40 transformation on the basis of the higher frequency of extensive damage to individual cells in the SV40 population. Adenovirus 12 HK cells were the most stable, and adenovirus 2^{+t7} HK-1 cells the least stable in maintaining the X chromosome in the karyotype.

DISCUSSION

From the data presented, it is apparent that the various adeno-SV40 hybrid viruses transform WHK tissue culture cells. It appears that the SV40 genome or the portion present in the hybrid particle, plays the major role in determining the characteristics of cells transformed with the adeno 2–, adeno 3–, and adeno 7–SV40 hybrid viruses since the morphology of the transformed cells and tumors induced with these cells (9), the chromosomal aberrations, and the presence of the SV40 T antigen all resemble the transformation of WHK by SV40 previously described (15, 20–22).

Several lines of evidence are present, however, which indicate that the adeno genome may also be permanently associated with the transformed cell. Some hamsters bearing tumors induced with Ad. 7^{+}–transformed cells developed antibody to the adeno 7 T antigen, despite the fact that the adeno 7 T antigen could not be demonstrated in the transformed cells or in the tumors. Preliminary fluorescent antibody–staining reactions carried out with sera from animals bearing tumors induced with Ad. 2^{+t7}– and Ad. 2^{++}–transformed cells indicate that these cells may contain early antigens coded for by the adeno 7 and 2 genomes respectively. Since a portion of adeno 7 DNA, transferred with the SV40 genome from the Ad. 7^{+} hybrid during the transcapsidation process (6) is present in the Ad. 2^{+t7} hybrid, it appears that the transforming genome was composed of SV40 and adeno 7 DNA. In addition, common adenovirus antigens, perhaps viral in nature, are also present in Ad. 2^{+t7}– and Ad. 2^{++}–transformed cells as evidenced by the staining with the hyperimmune ascites fluids. Finally, tumors produced in hamsters by Ad. 2^{+t7}– and Ad. 2^{++}–transformed cells have areas that resemble adenovirus-induced tumor or tumors induced with adenovirus 12–transformed cells (9). Thus, preliminary evidence indicates that part of the genome of a nononcogenic adenovirus may be integrated in the transformed cell with the production of an early antigen having the properties of a T antigen. It should be emphasized that the tumors and transformed cell lines were free of detectable infectious adenovirus and the sera from tumor-bearing hamsters contained no viral CF antibody and, in the sera from hamsters bearing tumors induced with Ad. 2^{+t7}– and Ad. 2^{++}–transformed cells, no neutralizing antibody as well.

The Ad. 12^{+t7} hybrid virus transformed WHK cells more reproducibly and with a shorter latent period than adeno 12. Simultaneous infection of WHK

cultures with both SV40 and adeno 12 resulted in the largest number of transformed foci, and the shortest latent periods. Thus the SV40 genome enhanced the oncogenic potential of adenovirus 12. A similar type of enhancement has been found in vivo; tumors developed with much shorter latent periods and in a larger number of hamsters inoculated with a mixture of adenovirus 12 and SV40 which had been passed in AGMK cultures than with either virus alone (23). Presumably, an adeno 12-SV40 hybrid virus was formed during such passage (23).

Enhancement of adenovirus infectivity in AGMK cultures by SV40 virus has also been described (24). Although the mechanism of this enhancement is not clear at present, it has been shown that the block in adenovirus replication in AGMK cells occurs subsequent to adenovirus T antigen formation and DNA synthesis (25), perhaps either transcription or translation of late messenger RNA; with SV40 DNA present, this block is presumably overcome. Whether the enhancement in AGMK cells is similar to the enhancement of adeno 12 transformation described here is not known at present. There is evidence that the SV40 genome in the Ad. 12^{+t7} hybrid has become integrated in the transformed cell since virtually every cell contains the SV40 T antigen and in larger amounts, as determined by CF, than are usually encountered in SV40-transformed cell extracts. Moreover, nuclei of the transformed cells and the chromosomal aberrations bear a closer resemblance to SV40-transformed cells than they do to cells transformed by adeno 12. Thus, although the morphology of the Ad. 12^{+t7} HK cells and the tumors induced with these cells (9) are similar to the Ad. 12 HK cells, evidence exists that the SV40 genome is also operative in these cells.

Most of the cytogenetic findings on adenovirus-induced tumors have revealed a tendency to form a near diploid modal number of chromosomes. Although some aneuploidy, chromatid breaks, and aberrant chromosomes were observed (26, 27) the cells containing such chromosomes were of low frequency, except in one study (28). SV40 transformation, on the contrary, results in an unstable karyotype with marked aneuploidy, polyploidy, and structural chromosome aberrations (21, 29, 30). It should be noted, however, that the adenovirus studies have been done with tumor cells while the SV40 studies have been carried out with in vitro transformed cells.

The adeno 12–transformed cell line described here contained cells with chromosomes which had a clear modal distribution in the diploid range and a constant karyotype. This is consistent with earlier studies. The cell lines derived from transformations induced by the Ad. 2^{+t7}, Ad. 2^{++}, Ad. 7^{+}, and Ad. 12^{+t7} hybrid preparations contained cells with an unstable karyotype, a high percentage of which had marked chromosome aberrations. It thus appears that the presence of the SV40 genome in the transformed cell confers the karyotypic instability characteristic of SV40 transformation.

SUMMARY

Primary weanling hamster kidney cultures were transformed with the adeno 2–SV40, adeno 3–SV40, and adeno 12–SV40 hybrid viruses and with adenovirus type 12. The transformed cell lines which were established were characterized with respect to morphology, virus and antigen content, and chromosome aberrations. The adeno 2 and adeno 3–SV40 hybrid transformed cells had the morphology and T antigen content characteristic of SV40 transformations; cells transformed by the former hybrid had cytogenetic changes typical of SV40-transformed cells as well. The adeno 12–SV40 transformed cells were similar morphologically to adeno 12–transformed cells, contained both the SV40 and adeno T antigens and demonstrated the karyotypic instability of SV40-transformed cells, indicating that both viral genomes are operative in these cells. Although the results indicate that the SV40 genome in hybrids derived from the moderately or nononcogenic adenoviruses supplies the determinants for most of the characteristics investigated, and perhaps for oncogenesis, evidence was presented which suggests that a portion of a nononcogenic adenovirus genome may be integrated in adeno 2–SV40 transformed cells and directs the synthesis of adenovirus T antigens.

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