Studies of Nondefective Adenovirus 2-Simian Virus 40 Hybrid Viruses

Transformation of Hamster Kidney Cells by Adenovirus 2 and the Nondefective Hybrid Viruses

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Nonhybrid adenovirus 2 (Ad2) and five nondefective Ad2-simian virus 40 (SV40) hybrids (Ad2⁺ND₁-Ad2⁺ND₅) failed to produce tumors when injected into newborn hamsters. However, each of these agents transformed hamster kidney cells in vitro, and the transformed cell lines produced tumors when injected into suckling hamsters. Foci of cells transformed by the hybrids could not be distinguished from foci of cells transformed by nonhybrid Ad2. Histopathologically, tumors induced by the nondefective hybrid-transformed lines were of the adenovirus type with no areas of SV40-type fibrosarcoma. All of the cell lines studied contained Ad2 T antigen and Ad2 RNA. Only the B55HK₁ line contained the three early SV40 antigens, U, TSTA, and T. The ND₄HK₁ line contained SV40 T and TSTA antigens (the latter antigen being detected only after 48 to 49 tissue culture passages) and SV40 RNA. The ND₅HK₁ line contained SV40 RNA but no detectable SV40 antigens; the other hybrid-transformed lines contained no SV40 RNA. The transformation of hamster cells by the nondefective Ad2-SV40 hybrids seemed to be caused by the Ad2 genome and could not be attributed to the regions of the SV40 genome contained within the hybrids. Additionally, these results indicate that interactions between the genomes of the nondefective hybrid viruses and the hamster cells they transform are complex and could involve the site of integration, the size of the incorporated viral genome, the transcription or translation of the viral genome, or various combinations of these processes.

Five nondefective adenovirus 2 (Ad2)-simian virus 40 (SV40) hybrids have been isolated and characterized (34, 35). Three of these agents induce one or more early SV40 antigens, and all induce early SV40 RNA (Table 1; 32). Each contains a unique piece of SV40 DNA covalently linked at the same location on the Ad2 genome (10, 22, 27, 30). These pieces of SV40 DNA represent portions of the SV40 DNA segment which maps between 0.11 and 0.59 SV40 units from the site on the SV40 genome cleaved by the R1 restriction endonuclease of *Escherichia coli* (Table 1; 42).

SV40 produces tumors when injected into newborn hamsters and transforms hamster cells in tissue culture (1, 5, 12, 17, 45, 48). Cell lines established from SV40-transformed cultures readily produce tumors when injected into newborn hamsters (1, 4). Nonhybrid Ad2, however, is not oncogenic in newborn hamsters and has not been reported to transform hamster cells in vitro (20). Although this virus does transform

rat embryo cells in vitro (13), these transformed cells fail to produce tumors when injected into newborn rats (39).

The early regions of the SV40 genome which are contained within three of the nondefective Ad2-SV40 hybrids appear to be closely associated with the oncogenic properties of SV40 in that the antigens induced by these regions are expressed in SV40 tumors and transformed cells (7, 18, 28, 36, 49). Consequently, we were interested in determining whether the integration of these segments of the SV40 genome into the Ad2 genome altered the ability of Ad2 to produce tumors in newborn hamsters and transform hamster cells in tissue culture. We were unable to induce tumors in newborn hamsters with Ad2 or any of the nondefective hybrids. However, we were successful in transforming hamster kidney cells in vitro with nonhybrid Ad2 as well as with each of the five nondefective Ad2-SV40 hybrid viruses. The purpose of this report is to describe the transformation of

hamster kidney cells by these viruses and to characterize some of the cell lines established during these studies.

MATERIALS AND METHODS

Viruses. The isolation and characterization of Ad2 (strain Ad2⁻), the nondefective B55 hybrid pool, and the five nondefective Ad2-SV40 hybrid viruses used in these studies have been described (27, 34, 35). Pools of viruses were prepared in human embryonic kidney (HEK) cells or African green monkey kidney cells (AGMK, used for B55 and Ad2⁺ND₄) and represented the second passage after plaque purification (Ad2⁺ND₁, Ad2⁺ND₂, Ad2⁺ND₃, and Ad2⁺ND₅) or plaque isolation (Ad2⁻, B55, and Ad2⁺ND₄). The infectivity titer of the B55 hybrid pool was 10^{6.1} PFU/ml by plaque titration in HEK cells. By similar tests, infectivity titers of the other pools ranged between 10^{7.1} and 10⁹ PFU/ml.

The propagation of Ad2 (strain Ad6) in HEK cells and of SV40 strain 777 by low-multiplicity passage in BSC-1 cells has also been described (3, 34). The infectivity titers of the pools of Ad2 used in these experiments varied between 10^{8.2} and 10^{9.3} PFU/ml, whereas the infectivity titers of the SV40 pools varied between 10^{8.7} and 10^{8.9} PFU/ml.

Transformation of hamster kidney cells. Cultures of primary hamster kidney cells were prepared by trypsinization of organs from 20- to 30-day-old hamsters from the NIH colony. Seeded in plastic dishes (100 by 20 mm), these cultures were maintained until confluent with Eagle minimal essential medium (EMEM) containing penicillin (250 U/ml), streptomycin (250 µg/ml), and glutamine (2 mM) supplemented with 10% fetal bovine serum (EMEM-10). Before infection, all cultures were washed and refed with EMEM containing 0.1 mM calcium, 2 mM glutamine, penicillin (250 U/ml), and streptomycin (250 µg/ml) supplemented with 5% dialyzed calf serum and 2% fetal bovine serum (low-calcium EMEM) (14). Ten cultures were infected with concentrations of either nonhybrid Ad2⁻ or the nondefective Ad2-SV40 hybrid viruses varying between 0.1 and 20 PFU/cell. When cytopathic changes (CPE) involved 50 to 75% of the monolayer (about 3 days after infection), all cultures were washed two to three times with low-calcium EMEM and refed with additional low-calcium EMEM containing 2 to 4 U (35) of Ad2 antiserum per ml. Cultures were maintained on antiserum-containing medium for 14 to 21 days in an attempt to retard CPE. Subsequently, the cultures were fed with low-calcium EMEM without Ad2 antiserum.

To transform hamster kidney cells with SV40, cell suspensions (10⁶ cells/ml) in low-calcium EMEM were infected with 10 to 100 PFU per cell and gently agitated for 3 h at 37 C. After being plated in plastic dishes (150 by 25 mm), the cells were maintained on low-calcium EMEM for about 5 weeks, at which time cell lines were established from transformed cultures.

Cocultivation and Sendai fusion tests for infectious virus. To screen transformed cell lines for release of infectious Ad2 or SV40 by cocultivation with sensitive indicator cells, 10⁶ cells from each line

were seeded onto each of 10 tube cultures of HEK or AGMK cells. The mixed cultures were observed and refed biweekly for 3 to 4 weeks. If no CPE developed, the cultures were disrupted by scraping and freeze-thawing, and samples of the cells and fluids were blind-passed to fresh tubes. These cultures were observed for another 3 to 4 weeks. If no CPE was apparent after blind passage, the transformed line was considered to be negative for infectious virus by cocultivation.

Fusion of transformed cells with indicator cells was performed by the technique of Gerber (16). For these tests, 5 × 10⁶ tumor cells and 5 × 10⁶ HEK or AGMK cells were mixed with 1 ml of a UV-inactivated Sendai virus suspension containing 40,000 hemagglutinating units (provided by K. K. Takemoto). The mixture was incubated 10 min at 4 C followed by a 20-min incubation at 37 C. The mixed suspensions were then washed once and dispensed into roller tubes (10⁶ cells/tube). The next day each culture was washed three times and refed with EMEM-10. Cultures were maintained for 3 to 4 weeks, blind-passed, and evaluated as described for cocultivation experiments.

Immune sera and serological tests. The techniques for preparing and characterizing hyperimmune Ad2 neutralizing antisera, and the SV40 viral (V)-specific antiserum used for complement fixation (CF) testing, have been described (33, 36). Ad2 T antisera were obtained from weanling hamsters carrying large (40 to 60 mm) tumors induced by the Ad2HK₁ line (see Results) of hamster kidney cells transformed by the Ad2⁻ strain of Ad2. Sera used for serological testing of cell-pack and cover-slip antigens reacted at ≥1/160 dilutions with Ad2 T antigens by both CF and immunofluorescent (FA) tests and failed to react with Ad2 V antigen or neutralized Ad2 in plaque neutralization tests. The SV40 T⁺U⁺ and T⁺U⁻ pools of hamster sera have been described (36).

The microtiter CF test and the indirect FA procedure used for staining cover-slip preparations of infected and transformed cells have been described (24, 43).

Antigens. The techniques for preparing infected cell-pack and cover-slip antigens to be used for CF and FA tests have been described (33, 36). Cytosine arabinoside (CA) was used to inhibit the development of V antigens in cell-pack and certain cover-slip preparations of Ad2 T and SV40 T antigens. By CF testing (using sera from hamsters bearing tumors induced by Ad2-transformed hamster cells; see Results), Ad2 cells-pack antigens had Ad2 T antigen titers of ≥1:64, whereas Ad2 V antigen titers were <1:4. SV40 cell-pack antigens tested by similar techniques had SV40 T titers ranging between 1:32 and 1:128, and SV40 V titers were <1:8. To determine the SV40 U antigen titer in SV40 cell packs, 0.2-ml samples were heated at 50 to 56 C for 30 min and tested by CF, using T⁺U⁺ and T⁺U⁻ sera from hamsters bearing tumors induced by SV40-transformed hamster cells (36). Heat-stable U antigen titers in these cell packs ranged between 1:8 and 1:32. After heating, these SV40 cell packs failed to give significant reactions (3 to 4+) at 1:2 dilutions with 4 to 8 units of T⁺U⁻ hamster sera. The SV40 antigenic properties of the

transformed human cell line, W18 VA2, have been described (36).

Ad2 V antigen was prepared from a pool of Ad2 grown in a suspension culture of KB cells, purified by banding in CsCl, dialyzed overnight against 0.01 M Tris-buffered saline, and stored at -70°C . The sample of suspended virions used as antigen had a titer of 1:320 when tested by CF against 4 to 8 units of Ad antibody in a convalescent serum. The SV40 V antigen used in these experiments has been described (36).

Transplantation rejection tests. To determine whether nondefective Ad2-SV40 hybrid-transformed cell lines contained SV40 transplantation rejection antigen (TSTA), groups of weanling hamsters (5 to 10 animals per group) were given intraperitoneal injections at weekly intervals of 2×10^6 to 3×10^6 transformed cells which had been treated with 2,500 rads of X irradiation (18) by using a 250 KVCP Westinghouse Dual Quadroconex unit. One week after the third immunization, hamsters were challenged by subcutaneous injection of $10^{1.1}$, $10^{1.6}$, and $10^{2.1}$ tumor-producing doses of the previously described THK-1₁ line of SV40-transformed hamster cells (37). Controls consisted of uninoculated hamsters and animals inoculated with 2×10^6 to 3×10^6 X-irradiated THK-1₁ cells.

Nucleic acid extraction and RNA-DNA hybridization. To obtain radiolabeled RNA from the transformed hamster cells, confluent monolayers in 32-oz (about 960 ml) prescription bottles were refed with 15 ml of medium containing 30 μCi of each of three tritiated nucleotides (New England Nuclear Corp.; [$5\text{-}^3\text{H}$]uridine, >20 Ci/mmol; [$2,8\text{-}^3\text{H}$]adenosine, 20 to 30 Ci/mmol; and [$5\text{-}^3\text{H}$]cytidine, 15 to 30 Ci/mmol) per ml. The cells were harvested by scraping 24 h after labeling, and total cell RNA was extracted by a hot phenol-sodium dodecyl sulfate (SDS) procedure (31, 32) and stored at -30°C in $2 \times \text{SSC}$ ($\text{SSC} = 0.15$ M sodium chloride plus 0.015 M sodium citrate, pH 6.9) plus 0.05% SDS. RNA concentrations were determined by the orcinol reaction (8) with yeast-soluble RNA (Calbiochem) as a standard.

The procedures used to extract DNA from purified Ad2 and SV40 virions and to hybridize [^3H]RNA with single-stranded viral DNA immobilized on nitrocellulose membrane filters have been described (31, 32). To perform elution-rehybridization experiments, filters were removed from scintillation fluid after the initial round of hybridization, dried, and eluted twice with $0.01 \times \text{SSC} + 0.05\%$ SDS at 90°C for 15 min. Eluates were pooled, brought to a final concentration of $2 \times \text{SSC} + 0.05\%$ SDS, and hybridized with fresh DNA-containing filters.

RESULTS

Attempted tumor induction in newborn hamsters and transformation of hamster cells in tissue culture. To determine whether the integration of regions of the early SV40 genome into Ad2 DNA changed the nononcogenic characteristics of Ad2 virions, groups of newborn NIH hamsters (40 to 65 animals per

group) less than 24 h old were injected subcutaneously with $10^{7.1}$ to $10^{7.7}$ PFU of four of the five nondefective Ad2-SV40 hybrids. In similar tests, newborn hamsters were injected with 10^5 PFU of the B55 hybrid pool and $10^{8.1}$ PFU of nonhybrid Ad2⁻. Of 318 inoculated hamsters, 183 survived and were observed for periods of 216 to 620 days with no gross evidence of tumor induction by any of these viruses (Table 1).

In view of these negative results, we next attempted to determine whether nonhybrid Ad2⁻ or any of the five nondefective Ad2-SV40 hybrids would transform hamster cells in vitro. Primary hamster kidney cells were infected as described in Materials and Methods and observed for the development of transformed foci. During the first 4 to 5 days after inoculation, CPE relentlessly destroyed the monolayer in spite of the presence of Ad2 antibodies in the surrounding medium. Despite nearly total cell destruction, foci (usually one to three per dish) of transformed cells appeared in several dishes in most tests 3 to 5 weeks after infection. Initially, the foci appeared as tightly packed clusters of fibroepithelial cells which tended to enlarge by growth at the periphery (Fig. 1). Evidence of loss of contact inhibition such as piling up and seeding of the surrounding dish developed 1 to 4 weeks later. Many of the initial colonies contained one or more large multinucleated giant cells. Although variations in the appearance of foci and the size of the transformed cells were noted, these variations seemed nonspecific. We were unable to distinguish between colonies of cells transformed by hybrid or nonhybrid viruses by their morphology (Fig. 1).

To establish lines of nonhybrid- and hybrid-transformed cells, dishes containing transformed foci were maintained for 2 to 6 months, until secondary growth by the original foci had established a dense culture. Such cultures were disrupted with trypsin-EDTA and established in 75-cm² plastic flasks. After four to five passages in plastic flasks, the cell lines were transferred to 32-oz (about 960 ml) glass prescription bottles. At this time the medium was changed from low-calcium EMEM to EMEM-10, with no noticeable effect on the growth of the transformed cells.

During the 2- to 6-month period when the colonies were growing in culture dishes, a "crisis" developed in several experiments during which the cells rounded up and floated in the media. After a 2- or 3-week period in some cultures undergoing crisis, new colonies were formed, and cell lines were eventually established from these cultures. Other degenerated

TABLE 1. Comparison of the properties of the nondefective Ad2-SV40 hybrid viruses with nonhybrid Ad2 and SV40

Virus	SV40 antigen induction ^a				Competition (%) between hybrid virus RNA and late SV40 RNA ^b	Size of SV40 DNA segment (SV40 units) ^c	Induction of tumors in newborn hamsters	Transformation of hamster cells in tissue culture
	U	TSTA	T	V				
Ad2							0	+
Ad2 ⁺ ND ₁	+	0	0	0	18	0.18	0	+
Ad2 ⁺ ND ₂	+	+	0	0	31	0.32	NT ^d	+
Ad2 ⁺ ND ₃	0	0	0	0	3	0.06	0	+
Ad2 ⁺ ND ₄	+	+	+	0	43	0.48	0	+
Ad2 ⁺ ND ₅	0	0	0	0	25	0.28	0	+
B-55	+	+	+	0			0	+
SV40	+	+	+	+	100	1.0	+	+

^a U, T, and V antigen induction was determined by CF testing (36). Induction of SV40 TSTA was determined by the rejection of tumors by immunized hamsters (37).

^b From the data of Levine et al. (32).

^c Determined by heteroduplex mapping techniques (27, 42).

^d NT, Not tested.

FIG. 1. Transformed colonies of hamster kidney cells. (a) and (b) Colonies transformed by Ad2⁻; (c) colony transformed by Ad2⁺ND₄. Magnification, $\times 25$ to 35.

cultures failed to regrow.

Uninfected samples of the same lots of hamster kidney cells served as controls for these experiments. These control cultures (carried in parallel with infected cultures in every transformation test) deteriorated rapidly with rounding-up of individual cells and retraction of the monolayer beginning within 4 to 7 days after plating. Any cells remaining in control dishes were trypsinized when the transformed cell lines were initiated. None of the control cells from any of these tests survived the initial passage.

The results of these experiments indicate that the nondefective Ad2-SV40 hybrids tested, the B55 hybrid pool, and nonhybrid Ad2 failed to produce tumors when inoculated into newborn hamsters. However, each of these agents was

capable of transforming hamster kidney cells in tissue culture (Table 1).

Induction of tumors in hamsters by transformed cell lines. To identify the various lines of transformed hamster kidney cells, each was designated by the transforming virus (Ad2⁻, ND₁, ND₂, etc.) and by the sequence in which the lines were established from the cultures transformed by that virus. Thus, the Ad2-HK₁ line represents the first Ad2⁻-transformed hamster kidney line established in continuous passage. The three lines of ND₂-transformed hamster cells (Table 2) originated from different culture dishes; therefore, each of these lines represents an independent transformation event. To begin to characterize the lines of hamster kidney cells transformed by Ad2⁻,

SV40, and the nondefective Ad2-SV40 hybrids, the ability of these lines to induce tumors in animals was studied by injecting 10^6 to 10×10^6 cells subcutaneously in newborn hamsters. Two to twelve weeks after inoculation, tumors appeared in 5 to 83% of surviving animals (Table 2). Randomly selected tumors induced by each cell line were excised and minced, and fragments were injected into fresh litters of newborn hamsters for subpassage. With the exception of tumors induced by the ND₃HK₁ line, which were lost due to epidemic diarrhea in the hamsters, each of the tumor lines was carried for five passages in newborn hamsters in order to ensure optimal adaptation. After the fifth newborn passage, each tumor line was passed in weanling hamsters. When the tumors in weanling animals reached 40 to 60 mm in diameter, the hamsters were bled from the orbital sinus and the sera were tested for the presence of virus-specific antibodies.

During each of the five passages in newborn hamsters, portions of the excised tumors were fixed in 10% acetate-buffered Formalin, sectioned, and stained with hematoxylin-eosin for microscopic examination. Sections from each tumor were examined as unknowns and scored for presence of "typical" SV40-type and adenovirus-type histologies (6, 11, 23, 29). The tumors

produced by the SV40 HK₁ cells were all easily identified and contained mixtures of fibrosarcoma and adenocarcinoma. The tumors induced by the Ad2-HK₁ line were all small-cell, undifferentiated, malignant tumors similar to those produced in vivo by Ad12 and 7 (2, 23). In addition, they contained varying amounts of adenocarcinoma mixed with the small, undifferentiated cell areas, but they did not contain areas of fibrosarcoma similar to those seen in the SV40 tumors. All of the tumors produced by the five nondefective hybrid-transformed lines were of the adenovirus type, with no areas of SV40-type fibrosarcoma (2, 23, 25).

Colony formation in soft agar and tests for the release of infectious virus by transformed cell lines. Cells from transformed lines were tested for their ability to form colonies in soft agar by slight modifications of the method of Macpherson and Montagnier (38). Suspensions of cells in 0.33% agar containing 700, 3,000, and 12,500 cells per ml were plated on a 0.5% agar base. Cultures were maintained by feeding with half-strength EMEM-10 at 10- to 12-day intervals, and colonies were counted 4 to 6 weeks after plating. Cells from each of the lines tested produced colonies with efficiencies varying between 0.2 and 0.9% (Table 2). Uninfected hamster kidney cells included as controls in these

TABLE 2. *Properties of hamster kidney cell lines transformed by AD2⁺ and the nondefective Ad2-SV40 hybrid viruses*

Cell line	Induction of tumors in newborn hamsters (% of surviving hamsters with tumors)		Colony formation in 0.33% agar (% colony-forming cells) ^a		Recovery of infectious virus by cocultivation/cell fusion ^b		
	Passage tested ^c	%	Passage tested ^c	%	Passage tested ^c	Ad2	SV40
Ad2 ⁻ HK ₁ ^d	7	36	11	0.2	8, 9, 11	+ / + ^e	0/0
ND ₁ HK ₁	7	18	5	0.6	17, 41	0/0	0/
ND ₂ HK ₁	6	20	33	0.2	33	/0	
ND ₃ HK ₁	4	17			4	/0	
ND ₄ HK ₁	5	15			5	/0	
ND ₅ HK ₁	7	5	9	0.6	8, 12, 32	0/0	0/
ND ₆ HK ₁	12	40	6	0.3	23, 28	0/0	0/0
ND ₇ HK ₁	32	83	48	0.2	19, 24, 43	0/0	/0
B55 HK ₁	10	40	9	0.9	21	0/0	/0
SV40 HK ₁	6	60	8	0.5	6		+ /
THK-1 ₁		100			6		+ /
Normal hamster kidney cells				0.0			

^a Obtained from the ratio of the number of colonies formed per 3,000 cells plated.

^b Tests for Ad2 were done with HEK cells; tests for SV40 were done with AGMK cells.

^c Subpassage in tissue culture.

^d Ad2⁻ refers to virus inducing the transformation, and HK₁ designates the cell line as the first Ad2⁻ hamster kidney line established.

^e Infectious Ad2 virions were also present in cell-free supernatant fluids from these passage levels.

tests failed to produce colonies in 0.33% agar.

To determine whether the transformed cell lines yielded infectious adenovirus, they were tested at one or more passage levels by fusion with HEK (for Ad2) or AGMK (for SV40) cells by using UV-inactivated Sendai virions (16). In addition, some lines were tested by cocultivation of tumor cells with either HEK or AGMK (Table 2). The data indicate that none of the lines of nondefective Ad2-SV40 hybrid-transformed hamster cells produced infectious Ad2 or SV40 by fusion or cocultivation with sensitive indicator cells, whereas both the SV40 HK₁ and THK-1₁ lines produced SV40 by cocultivation with AGMK cells. Unexpectedly, the Ad2⁻ HK₁ line produced Ad2 (identified by neutralization with Ad2-specific antisera) by both cocultivation and Sendai fusion with HEK cells. Additional studies on cell-free supernatant fluids obtained from Ad2⁻ HK₁ cultures at the ninth through eleventh passages also contained infectious Ad2. Thus, it appears that the Ad2⁻ HK₁ line, in addition to being transformed, is either a carrier culture or (less likely) induces small amounts of infectious virus. Studies further evaluating this phenomenon are in progress.

Presence of virus-specific antigens in the transformed cell lines. Multiple approaches were used to detect virus-specific antigens in the cell lines established from hamster kidney cells transformed by Ad2⁻ and the nondefective Ad2-SV40 hybrid viruses; cell-pack and cover-slip antigens representing each cell line were tested by CF and FA by using specific antisera;

sera from hamsters bearing large (40 to 60 mm) tumors were tested by CF and FA against known virus-specific antigens, and certain cell lines were tested after X irradiation (18) for their ability to induce SV40 TSTA in weanling hamsters (Tables 3-5). The SV40-transformed hamster kidney cell line, THK-1₁, was included for comparative purposes in each of these experiments.

Each of the cell lines transformed by either Ad2⁻ or the nondefective hybrid viruses contained detectable concentrations of Ad2 T antigen (Table 3). Both the ND₁HK₁ and B55HK₁ cell lines contained SV40 T antigen. Unexpectedly, the heat-stable SV40 U antigen was detected only in the B55HK₁ cell line even though the Ad2⁺ND₁, Ad2⁺ND₂, and Ad2⁺ND₄ viruses induce readily detectable quantities of this antigen during lytic infection (35, 36).

In the Ad2⁻ and nondefective Ad2-SV40 hybrid-transformed cells stained by the indirect FA test using Ad2 T specific sera, Ad2 T antigen was distributed throughout the nuclei in a finely stippled, dustlike pattern that was weakly reactive (1+ or less on a 4+ scale). Many cells contained a single cytoplasmic flecklike body, morphologically similar to those described in Ad12 tumor cells (44). The minuteness of the staining particles and their lack of intensity made FA study of transformed cells difficult. These studies had to be carefully controlled and done repetitively with more than one serum for complete evaluation.

The reactions of sera from hamsters carrying

TABLE 3. *Antigenic characteristics of hamster kidney cell lines transformed by Ad2, SV40, and the nondefective Ad2-SV40 hybrid viruses*

Cell line	Passage tested ^a	CF titer					Antigenic characteristics by FA		
		SV40 U ^b	SV40 T, U ^c	SV40 V	Ad2 T	Ad2 V	Passage tested ^a	% positive	
								SV40 T, U ^c	Ad2 T
ND ₁ HK ₁	5		0 ^d	0	2	0	42	0	>90
ND ₂ HK ₁	34			0	32	0	34	0	
ND ₂ HK ₂	4		0	0	≥64	0			
ND ₂ HK ₃	5		0	0	≥64	0			
ND ₃ HK ₁	8		0	0	8	0	33	0	
ND ₄ HK ₁	5, 49	0	16	0	≥64	0	49	>90	>90
ND ₅ HK ₁	10, 47		0	0	16	0	47	0	>90
B55 HK ₁	10, 43	4	32	0	16	0			
Ad2 ⁻ HK ₁	9		0	0	16	0	8	0	>90
THK-1 ₁		16	64	0	0	0		>90	0

^a Subpassage in tissue culture.

^b Antigen heated 50 C for 30 min before CF testing.

^c Determined by serological reaction with antisera containing 4 to 8 CF units of both anti-SV40 T and anti-SV40 U antibodies.

^d 0 indicates a titer less than 1 to 2.

TABLE 4. *Viral-specific antibodies in the sera of hamsters carrying tumors induced by Ad2, SV40, and nondefective Ad2-SV40 hybrid-transformed hamster cells*

Cell line inducing tumors	Passage level ^a	CF antibody response to:						FA antibody response to:	
		SV40 hamster tumor extract (SV40 T)	SV40 in AGMK with CA (50 C 30 min) (SV40 U)	SV40 in AGMK with CA (SV40 T, U)	SV40 virions (SV40 V)	Ad2 in HEK with CA (Ad2 T)	Ad2 virions (Ad2 V)	W18 VA2 (SV40 T, U)	Ad2 in HEK with CA (Ad2 T) ^b
Ad2 ⁻ HK ₁	7	0/11 ^c		0/10	0/11	11/11 ^b	0/11	0/5	5/5
ND ₁ HK ₁	7	0/12			0/19	14/19 ^d	0/19	0/15	5/5
ND ₂ HK ₁	6			0/20	0/20	2/20 ^d	0/20	0/20	20/20
ND ₂ HK ₂	4			0/13	0/13	0/13	0/13	0/12	11/11
ND ₂ HK ₃	5			0/12	0/12	7/12 ^d	0/12	0/12	11/11
ND ₄ HK ₁	12	14/15	0/15	13/15 ^b	0/15	14/15	0/15	5/5	
ND ₅ HK ₁	32					2/27 ^d	0/35	0/31	29/31
B55 HK ₁			2/9 ^d	9/9 ^b	0/9	8/9 ^b	0/9		5/5
THK-1 ₁		5/5			0/5			5/5 ^b	

^a Subpassage in tissue culture.^b Serum antibody titers varied between 1 to 10 and ≥ 1 to 160.^c Number positive/number tested. 0 indicates < 1 to 10.^d Serum antibody titers varied between 1 to 10 and 1 to 40.TABLE 5. *Effects of immunization with X-irradiated nondefective Ad2-SV40 hybrid-transformed hamster cells on the induction of tumors by THK-1₁ cells*

Immunizing cells	Passage tested ^a	TPD ₅₀ ^b (log ₁₀)		Resistance ^c	
		Expt 1	Expt 2	Expt 1	Expt 2
None		2.9			
THK-1 ₁		≥ 5.1	≥ 5.2	160	200
ND ₁ HK ₁	41	≤ 3.8		≤ 8	
ND ₂ HK ₁	33	≤ 3.9		≤ 10	
ND ₂ HK ₂	4	≤ 3.8		≤ 8	
ND ₂ HK ₃	5	≤ 3.8		≤ 8	
ND ₄ HK ₁	6, 7	≤ 3.9	≤ 3.8	≤ 10	≤ 8
ND ₄ HK ₁	48, 49	4.7	4.3	63	25
ND ₅ HK ₁	5	≤ 3.8		≤ 8	
B55 HK ₁	9	≤ 3.9			≤ 10
B55 HK ₁	42	≥ 5.0			125

^a Subpassage in tissue culture.^b TPD₅₀, Tumor-producing doses; calculated by the method of Reed and Muench (46).^c Resistance = TPD₅₀ in immune hamsters/TPD₅₀ in nonimmune hamsters = $10^{2.9}$.

tumors induced by Ad2⁻ and the nondefective Ad2-SV40 hybrid virus-transformed cell lines (with the exception of the ND₃HK₁ line) are presented in Table 4. These data support the data in Table 3 and indicate that all of these tumors induced the formation of Ad2 T antibody detectable by either CF or FA testing. Tumors produced by the ND₄HK₁ and B55HK₁ lines induced the formation of SV40 T antibody, whereas only the tumors induced by the B55HK₁ line of transformed hamster kidney

cells produced low levels of antibody which reacted with the SV40 U antigen.

Eleven of the 72 sera from hamsters carrying tumors induced by ND₂HK₁, ND₂HK₂, ND₂HK₃, and ND₅HK₁ were weakly reactive (antibody titers 1:10 to 1:40) by CF testing against Ad2 cell-pack antigens. Of the same sera, 71 of 73 reacted more strongly (Table 4) with Ad2 T cover-slip antigens by indirect FA testing. The reasons for the lack of CF reactivity of these Ad2 T antisera are unknown; however, it is interesting to note that sera from humans convalescent from adenovirus infections do not appear to react with Ad T antigens by CF testing but to react by FA (33). Such data suggest the presence of Ad2 T antigens and antibodies with different reactivities.

To determine whether contaminating agents could have affected the results of our serological tests, the Ad2-HK₁, ND₂HK₂, and ND₅HK₁ lines were tested between the 5th and 47th passage levels for a variety of rodent viruses by both CF and mouse antibody production tests (9). These lines were negative for GDVII virus, pneumonia virus of mice, reovirus type 3, Kilham rat virus, polyoma virus, Sendai virus, minute virus of mice, lactic acid dehydrogenase virus, lymphocytic choriomeningitis virus, and ectromelia. In addition, all of the transformed lines studied were free of mycoplasma by anaerobic culture on Hayflick medium (21).

To determine whether the nondefective hybrid-transformed cell lines contained detectable amounts of SV40 TSTA, weanling hamsters

were immunized by intraperitoneal injection of X-irradiated cells and challenged with tumor-producing doses of SV40-transformed THK-1₁ cells. After 2,500 rads of X irradiation, none of the cell lines tested survived more than 10 to 14 days in tissue culture, and no gross intraperitoneal tumors were noted in the 75 animals that were autopsied at the conclusion of the experiments (Table 5). Immunization with X-irradiated SV40 THK-1₁ produced a 160- to 200-fold increase in the tumor-producing doses by unirradiated THK-1₁ cells. Immunization of hamsters with X-irradiated ND₁HK₁, ND₂HK₁, ND₂HK₂, ND₂HK₃, and ND₂HK₁ produced no appreciable increase in resistance of hamsters to tumor induction by THK-1₁ cells. Immunization with early passage ND₁HK₁ and B55HK₁ also failed to induce a significant increase in resistance to tumor production by THK-1₁ cells. Unexpectedly, immunization with the 48th and 49th passage levels of the ND₁HK₁ line and 42nd passage level of the B55HK₁ line produced a significant increase in resistance (Table 5). Thus, some unknown factor appears to have converted SV40 TSTA-negative cells to TSTA-positive cells during serial tissue culture passage of the ND₁HK₁ and B55HK₁ transformed hamster cells.

Presence of virus-specific RNA in hamster cells transformed by the nondefective Ad2-SV40 hybrids. Whole-cell [³H]RNA was extracted from each line and reacted with filters containing 1 μg of either Ad2 or SV40 DNA. The net counts per minute bound to the two DNAs is presented in Table 6. Only the ND₁HK₁ and ND₂HK₂ lines contained readily detectable SV40 RNA, although certain other lines yielded equivocal results in the first round of hybridization. After elution and rehybridization to fresh SV40 DNA, however, no binding was detected with [³H]RNA extracted from these other lines. In contrast, [³H]RNA from all of the lines reacted significantly with Ad2 DNA.

Comparing the presence of viral-specific RNA with the detection of viral-specific antigens in these hybrid-transformed lines (Table 7), it appears that only the ND₁HK₁ line induces both SV40 antigens and RNA. Paradoxically, at low-passage levels the ND₂HK₂ line appears to induce no SV40 antigens but contains readily detectable quantities of SV40 RNA. The other hybrid-transformed lines appear to contain only Ad2 antigens and Ad2 RNA.

DISCUSSION

Studies on the oncogenicity of adenoviruses resulted in the organization of human adenoviruses into three subgroups. Depending upon

TABLE 6. Hybridization of RNA from hamster kidney cells transformed by the nondefective Ad2-SV40 hybrids with SV40 and Ad2 DNAs

Cell line	Passage tested ^a	[³ H]RNA inputs (× 10 ⁴)	Net ³ H counts/min bound to 1.0-μg filters ^b of:	
			SV40 DNA	Ad2 DNA
1809 line ^c		256	-45	8
ND ₁ HK ₁	7, 44	80	9	1,828
ND ₂ HK ₁	8, 13	160	43	293
ND ₂ HK ₂	7, 11	100	808	1,708
ND ₂ HK ₃	7	146	-53	1,402
ND ₂ HK ₁	34	85	70	408
ND ₁ HK ₁	26, 49	131	2,719	2,693
ND ₂ HK ₁	45	90	27	501

^a Subpassage in tissue culture. No significant differences in ³H counts per minute bound to SV40 and Ad2 DNA filters were noted at different passage levels.

^b 1,200 to 1,800 μg of RNA (specific activity, 5 × 10⁴ to 10 × 10⁴ counts per min per μg) were added to vials containing either Ad2 or SV40 plus *E. coli* DNA filters. The data are the average counts per minute from six filters of each DNA after subtraction of the average counts per minute bound to the *E. coli* blank (3 × 10⁻⁴% of input RNA). Elution-rehybridization of ³H counts per minute on all original SV40 filters was performed. Additionally, ³H counts per minute on Ad filters from experiments with 1809, ND₂HK₁, and ND₁HK₁ RNAs were eluted and rehybridized as a control. The average ³H counts per minute eluted from the original filters was 90%, the total input per filter in the second round was 50 to 1,500 counts/min. The average net ³H counts per minute rehybridized to SV40 filters was 89 and 194 for ND₂HK₂ and ND₁HK₁, respectively; all other RNAs yielded fewer than 5 net counts/min after rehybridization with SV40 filters. The average net ³H counts per minute rehybridized to Ad filters was 10, 64, and 505 for 1809, ND₂HK₁, and ND₁HK₁, respectively.

^c Spontaneously transformed hamster cells (31).

their ability to induce tumors in newborn hamsters, the viruses comprising these groups were characterized as "highly" oncogenic, "weakly" oncogenic, or "nononcogenic" (20). Since the genomes of the viruses (Ad12, 18) representing the highly oncogenic subgroup were markedly dissimilar from the genomes of the nononcogenic adenovirus types 1, 2, and 5 (15, 19, 20), one could postulate that these genetic differences were reflected in the oncogenic spectrum of these two subgroups. However, recent reports (13, 39, 50) demonstrating that Ad1 and 2 will transform rat embryo cells and that Ad5 will transform hamster cells appear to refute such a concept (40).

When our studies were begun, we considered Ad2 to be nononcogenic and were hopeful that

TABLE 7. *Presence of viral-specific antigens and RNA in hamster cells transformed by the nondefective Ad2-SV40 hybrid viruses*

Cell line	Viral-specific antigens				Viral-specific RNA	
	SV40 U	SV40 TSTA	SV40 T	Ad2 T	SV40	Ad2
ND ₁ HK ₁	0	0	0	+	0	+
ND ₂ HK ₁	0	0	0	+	0	+
ND ₂ HK ₂	0	0	0	+	+	+
ND ₂ HK ₃	0	0	0	+	0	+
ND ₃ HK ₁	0	0	0	+	0	+
ND ₄ HK ₁	0	+	+	+	+	+
ND ₅ HK ₁	0	0	0	+	0	+

^a Detected at high-passage levels only.

one or more of the SV40 segments acquired by the Ad2 genome during the formation of the nondefective hybrids would confer upon the Ad2 genome the ability to induce tumors in hamsters or transform cultured hamster cells. However, our data demonstrate that the acquisition of SV40 DNA segments representing up to 48% of the SV40 genome does not appear to alter the inability of Ad2 to produce tumors when injected into newborn hamsters. We must emphasize that these SV40 DNA segments include the regions responsible for the induction of the three SV40 antigens (U, TSTA, and T) most closely associated with SV40-induced tumors and transformation. Furthermore, like Ad5, nonhybrid Ad2 will transform infected hamster cells *in vitro*, and these transformed cells produce tumors when injected into newborn hamsters. Thus, the lack of oncogenicity of Ad2 and Ad5 for newborn hamsters would appear to be a host-dependent phenomenon rather than an inherent inability of these genomes to transform hamster cells.

In any event, these results imply that transformation of hamster kidney cells by the nondefective Ad2-SV40 hybrids cannot be attributed to portions of the SV40 genome. In addition, neither the morphology of the hybrid virus-transformed colonies nor the histology of the tumors induced by these cells provided any clues which would associate any of the regions of the SV40 genome contained within the hybrids with the transformation event.

Ad2 T antigen was the only viral-specific antigen detected consistently in nondefective hybrid-transformed cell lines. Likewise, all of these cell lines contained Ad2 RNA and induced tumors with histological features similar to tumors induced by nonhybrid Ad2-transformed cells. No tumors contained areas of SV40-type histology even though ND₄HK₁-induced tumors

contained SV40 T antigen as determined by the high titers of SV40 T antibody in their hosts' sera. Consequently, it appears that the Ad2 genome was most likely responsible for the transformation of hamster kidney cells by the nondefective Ad2-SV40 hybrids and was dominant over the SV40 genome in determining the morphology of the induced tumors. Deletions (ranging from 4.5 to 7.1% [27]) of Ad2 DNA in the region containing the inserted SV40 segments had no apparent effect on the Ad2 genome's ability to either transform hamster cells, induce Ad2 T antigen, or alter the histology of the tumor produced by the transformed cell line. Schnipper et al. (47) were unable to discriminate between the effects of the nondefective hybrid viruses and nonhybrid Ad2 in the induction of cellular DNA synthesis in either monkey or hamster cells. These findings indicate that expression of the Ad2 genome in nondefective hybrid-infected or -transformed cells interferes with the expression or the detection of certain SV40 functions which appear to be associated with the early region of the SV40 genome.

The Ad2⁺ND₁ and Ad2⁺ND₂ viruses express SV40 U and SV40 U and TSTA antigens, respectively, during lytic infection. Neither of these antigens were detected in the hamster kidney cells transformed by these viruses. The Ad2⁺ND₄ virus induces SV40 U, TSTA, and T antigen during lytic infection. Only SV40 T antigen was detected in early-passage levels of hamster kidney cells transformed by Ad2⁺ND₄, whereas SV40 TSTA antigen was detected in the ND₄HK₁ line at higher-passage levels. During lytic infection, the B55 hybrid also induces SV40 U, TSTA, and T antigens. B55 hybrid-transformed hamster kidney cells express all three of these antigens; however, the TSTA antigen was detected only after many tissue culture passages. The Ad2⁺ND₃ and Ad2⁺ND₅ hybrids fail to induce detectable SV40 antigens during lytic infection, and hamster kidney cells transformed by these agents contain no detectable SV40 antigens. Most of the SV40 antigen-negative lines transformed by the nondefective hybrids produce no detectable SV40 RNA; however, at low passage levels, the ND₂HK₂ cell line contains significant amounts of SV40 RNA but no detectable SV40 antigens, whereas the other two Ad2⁺ND₂-transformed cell lines contain no detectable SV40 RNA. These puzzling results indicate complex interactions between the genomes of the nondefective hybrid viruses and the hamster cells they transform. Such interactions could involve the site of integration, the size of the incorporated viral genome, the transcription or translation of the viral genome, or

various combinations of these processes.

Workers in several laboratories have attempted to develop techniques to introduce functionally defined segments of genetic material into mammalian cells (26, 41). For example, Jackson et al. (26) plan to use SV40 as a possible "vector" to transport known DNA sequences into the cell genome. For this purpose they developed a technique for covalently joining this genome with the *λ*dgal region of *Escherichia coli* (26). The studies reported herein indicate that the Ad2 genome can also be used as a means of transporting defined segments of a foreign genome into mammalian cells. However, our studies imply that due to the complexities of the interaction between mammalian genomes and foreign DNA, the use of carrier DNA to integrate known heterologous functions into cellular genomes does not guarantee expression of the incorporated information.

The expression of detectable levels of SV40 TSTA by high-passage but not by low-passage ND₄HK₁ and B55HK₁ cells deserves further comment. We have been unable to find a report of similar aberrations in the expression of SV40 TSTA in other lines of SV40-transformed cells. Since the challenging dose of THK-1₁ cells was threefold greater than that used in similar tests which determined the ability of the nondefective hybrid virions to induce SV40 TSTA (37), it is possible that the test was too insensitive to detect trace amounts of SV40 TSTA in the immune hamsters. However, the 10- to 20-fold increase in resistance reproducibly detected in THK-1₁- and B55HK₁-immune hamsters provided a suitable range to detect lower antigen concentrations. In addition to the possibility of defects in transcription of the integrated viral genome during early passages, it is possible that the TSTA antigen was present in the cell and either not expressed on the cell surface or expressed in an inactive or masked form. Since the ND₄HK₁ and B55HK₁ cell lines are not clonally derived, it is also possible that clones of cells containing different portions of the SV40 genome emerged during serial passage.

The findings in this report suggest that in addition to their usefulness for genetic studies on Ad2 and SV40, the nondefective Ad2-SV40 hybrids should also be useful probes for genetic studies on mammalian cells. By further analysis of the nondefective hybrid-transformed cells, we hope to elucidate some of the mechanisms by which hamster cells respond to the integration of foreign DNA. If the mechanisms which explain the peculiar expression of the SV40 DNA segments of the nondefective Ad2-SV40 hybrid

genomes in transformed cells can be determined, such information may play a significant role in understanding some of the basic processes of viral-induced carcinogenesis.

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