

# In Vitro Transformation by Adenovirus-Simian Virus 40 Hybrid Viruses

## IV. Properties of Clones Isolated from Cell Lines Transformed by Adenovirus 2-Simian Virus 40 and Adenovirus 12-Simian Virus 40 Transcapsidant Hybrid Viruses

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Received for publication 23 July 1969

Clones were isolated from hamster cells transformed by the adenovirus 2-SV40 and adenovirus 12-SV40 transcapsidant hybrid viruses. The clones were characterized with respect to their cytomorphology, virus and antigen content, and the histomorphology of tumors induced by transplantation of the clonal sublines to hamsters. Three different cellular and colonial morphologies were observed. Clones with an SV40 morphology gave rise to tumors predominantly with an SV40 histology, whereas clones with an adenovirus morphology produced typical adenovirus tumors upon transplantation of the transformed cells. Clones which had features of both SV40 and adenovirus transformed cells gave rise to "intermediate" and adenovirus tumors. The results indicate that multiple events occur during transformation and tumorigenesis by the transcapsidant virus populations and provide an explanation for the multiplicity of findings which have been reported with these virus populations.

Adenovirus (ad)-SV40 hybrid viruses formed by passage of various adenoviruses in rhesus kidney cultures which contained SV40 virus (passage hybrids) (10, 15, 25, 29) and by transcapsidation procedures (transcapsidant or transfer hybrids) (23, 28) have been described. The latter hybrids are formed by growing the passage (ad 7-SV40) hybrid preparation in cells coinfecting with other adenoviruses and then adding ad 7 antiserum. In this way, the portions of the original ad 7 and SV40 genomes contained within the defective hybrid particle are transferred to capsids supplied by the other adenoviruses (ad 2 and ad 12 in this case). The resultant hybrid population consists of two particles, complete ad 2 or ad 12 virions and a defective particle in an ad 2 or ad 12 coat; it is not known whether the defective particle, in addition to the ad 7-SV40 genomes, contains ad 2 or ad 12 genetic information as well.

In previous work, *in vitro* transformation of hamster kidney cells with various hybrid preparations was described (2, 5, 6, 11). The resultant cell lines obtained from these transformations were examined with respect to morphology, virus

and antigen content, cytogenetic aberrations, and the pathology of the tumors induced in hamsters by transplantation of the transformed cells. From these data, evidence was obtained that both ad and SV40 viral genomes were present in the population of transformed cells. However, as additional cell lines were isolated after transformations by the transcapsidant hybrid viruses, cells with morphological characteristics of both ad- and SV40-transformed cells were observed and mixed, or dimorphic tumors appeared upon transplantation of transformed cells to hamsters.

To determine whether mixtures of transformed cells were present and to establish more definitively which viral genomes are present in cells with different morphologies, clones were established from cells transformed by the ad 2 and ad 12 transcapsidant hybrid viruses. The results of an examination of some properties of these clones and the pathology of tumors induced by transplantation of the clonal lines to hamsters form the basis of this report. In the accompanying paper, more direct evidence on the results of nucleic acid hybridization studies, in which the

presence of the various viral genomes in the clones was demonstrated, will be presented (14).

These studies indicate the following. At least three different transformation events occurred with the transcapsidant hybrid viruses; the clones differ with respect to the nature and presence of the ad and SV40 genome(s); the morphology of the clones and tumors obtained by transplantation of the clones is determined by the viral genomes present; and the transcapsidant particle in the ad 2 and ad 12 populations is most likely composed of the ad 7 and SV40 genomes, covalently linked, in ad 2 or ad 12 capsids, respectively.

#### MATERIALS AND METHODS

**Virus and cell lines.** The origin and passage history of the ad 2-SV40 (ad 2<sup>+</sup>) and ad 12-SV40 (ad 12<sup>+</sup>) transcapsidant hybrid populations have been described (28); they were passed three to five times in African green monkey kidney (AGMK) cells followed by an additional three passages in the presence of ad 7 antiserum. The ad 2<sup>+</sup> passage line was further freed of ad 7 virus by a limiting dilution passage in AGMK (28). The transformed cell lines were established by trypsinization and passage of transformed weanling hamster kidney (WHK) cells as previously described (5, 6). The cell lines are designated by the transforming virus (ad 2<sup>+</sup>), the tissue transformed (hamster kidney, HK), and the number of the cell line established (Table 1). Seven cell lines were utilized in this study; the origin and properties of three of these (ad 2<sup>+</sup> HK-3, ad 2<sup>+</sup> HK-4, ad 12<sup>+</sup> HK-1) have been briefly described in a previous report (6).

**Cloning procedure.** The clonal sublines utilized in the present study were derived from two separate clonings with either of two cloning techniques (see Table 1). The majority of the cell lines were cloned by a modification of the agar suspension colony technique of MacPherson and Montagnier (18). The cells were dispersed with a trypsin-EDTA (ethylenediamine-tetraacetate) solution, and  $5 \times 10^4$ ,  $10^6$ , and  $2 \times 10^6$  cells were planted in soft agar (0.33%). The cells were observed to be monodispersed at the time of counting,

the clump rate being less than 1%. At 3 to 6 weeks, well-isolated colonies were picked with Pasteur pipettes and grown to mass cultures.

It was observed that cells having an ad morphology (see below) grew poorly in agar and the cloning of these cell lines in agar selected against this type of cell (ad 12<sup>+</sup> HK-3). Moreover, some cell lines grew poorly or not at all in agar (ad 12<sup>+</sup> HK-3, ad 12<sup>+</sup> HK-4). These lines were therefore cloned on plastic petri dishes by a modification of the method of Puck (21). Single cells were isolated by small stainless steel cylinders 1 to 2 days after plating 50 to 100 cells per dish; after sufficient growth the clones were trypsinized within the cylinder and established as previously described (4). Some cell lines where the presence of ad-transformed cells was suspected were cloned by both methods to insure that selection by the agar method had not occurred. The reason(s) for the differences in the growth of the various cell lines in agar is not known; the relatively poor growth of ad-transformed cells has been noted previously (17).

The cell lines were maintained in medium NCTC 109 with 10% fetal calf serum, 2 mM glutamine, penicillin (250 units), and streptomycin (250  $\mu$ g/ml). Parallel cultures of the ad 2<sup>+</sup> HK-4 cell line were maintained in the NCTC medium as well as medium containing 1.8 or 0.1 mM calcium (6) for 10 passages. All cultures were maintained in a stationary position at 37 C and the medium was changed twice weekly.

**Virus isolation.** All cell lines were tested for the presence of infectious ad and SV40 viruses by overlaying viable transformed cells on primary human embryonic kidney (HEK) and AGMK cultures, respectively, which were obtained from Microbiological Associates, Bethesda, Md. After 13 to 21 days, the dual cultures were frozen and thawed twice and the extracts blind-passed to fresh HEK and AGMK cultures. Details of these procedures have been described previously (5, 6). Attempts were not made to rescue the defective, SV40-containing component from transformed cells by using helper adenoviruses.

**T antigen detection.** The fluorescent-antibody (FA) technique was used to detect the presence of SV40 and ad T antigens in transformed cells. The cells were grown on cover slips, fixed in acetone, and stained by

TABLE 1. Derivation of clones

Cell line	Passage <sup>a</sup>	Methods of cloning	
		Agar	Plastic
Ad 2 <sup>+</sup> HK-3	12	+ (1, 3, 8, 10, 15) <sup>b</sup>	+ (12)
Ad 2 <sup>+</sup> HK-4	16	+ (1, 3, 6, 7, 8)	
Ad 12 <sup>+</sup> HK-1	10	+ (1, 3)	
Ad 12 <sup>+</sup> HK-2	13	+ (3, 4)	
Ad 12 <sup>+</sup> HK-3	9	+	+ (2, 5, 10, 12)
Ad 12 <sup>+</sup> HK-4	5	+	+ (1, 3)
Ad 12 <sup>+</sup> HK-5	7	+ (1, 6)	+ (10)

<sup>a</sup> Passage level at the time of cloning; all studies were carried out with clonal sublines at the 4th-7th passage levels.

<sup>b</sup> Numbers in parentheses indicate clones obtained by particular method of cloning.

the direct or indirect FA technique with pooled sera from hamsters bearing SV40- or ad-induced tumors and fluorescein-conjugated goat antihamster serum (6, 19, 20). Ad 12 T antigen was demonstrated by utilization of pooled sera from hamsters bearing tumors induced with ad 12-transformed hamster cells. Three separate pools of sera from hamsters bearing tumors induced with ad 7-transformed cells were utilized in an attempt to demonstrate the presence of ad 7 T antigen. One of these, obtained from Flow Laboratories, Rockville, Md., was directly conjugated with fluorescein isothiocyanate. Two types of sera were used in an effort to demonstrate ad 2 T antigen(s) since it has been demonstrated that ad 1 and ad 2 T antigens cross-react (3). The first type consisted of pooled sera from hamsters bearing transplanted tumors induced with the ad 1-SV40 hybrid virus (3). Since these sera also contained antibodies to the SV40 T antigen, and since the ad 2<sup>+</sup> hybrid-transformed cells contained the SV40 T antigen, they were adsorbed with SV40-transformed cells by methods previously described (27); despite five cycles of adsorption, the SV40 reactivity of these sera could not be eliminated. The second type of serum used was obtained from a patient convalescing from an ad 1 infection and was kindly provided by A. M. Lewis, Jr. (16); a horse anti-human conjugate was utilized in indirect FA tests with this sera. Both the hamster and human sera had titers of 1:80 to 1:160 when used to stain HEK cells infected with ad 2 in the presence of 10<sup>-8</sup> M 5-fluorodeoxyuridine. The ad 12 tumor serum pool was also used to stain the ad 2<sup>+</sup>-transformed cell lines in an attempt to demonstrate the presence of cross-reactive or group-reactive T antigens (see Table 2).

**Transplantation.** Cells (1 × 10<sup>6</sup> to 8 × 10<sup>6</sup>) from the various mass cultures and their derived clones were dispersed with a trypsin-EDTA solution, sedimented, suspended in phosphate buffered saline (pH 7.2), counted, and inoculated subcutaneously into newborn or weanling Golden Syrian hamsters (*Mesocricetus auratus*). The hamsters were observed weekly for the appearance of tumors which were harvested when they reached a mean tumor diameter of 2 to 6 cm; the tumors were then fixed in 10% Formol-saline or corrosive trichloroacetic acid solution and stained with hematoxylin and eosin.

## RESULTS

**Cytomorphology.** Three different cellular and colonial morphologies were present in the mass cultures derived from ad 2<sup>+</sup> and ad 12<sup>+</sup> transcapiscent virus-transformed hamster kidney cells and their derived clones; these included clones with the morphology of SV40-transformed cells, ad-transformed cells, and cells with an intermediate or mixed morphology, containing features of both SV40- and ad-transformed cells. (Fig. 1)

The SV40 type colony was round or irregularly shaped with cells migrating outward from the periphery of the colony. The cells were fibroblastic or triangular-shaped, frequently tended to

grow in whorls, and had a slight tendency to pile up in the central portions of the colony. The nuclei were large, pleomorphic, round to ovoid, and contained approximately 6 to 10 nucleoli as well as masses of coarse, clumped chromatin; the nuclear to cytoplasmic ratio was increased. Eosinophilic, cytoplasmic bodies were present in a small proportion of cells and multinucleated giant cells were present in nearly every colony (Fig. 1e and f).

The ad type colony was round or irregularly shaped and contained small, tightly packed epithelial cells with dense multilayering of the cells in the central portions of the colony. The nuclei, which filled practically the entire cell, were pale, pleomorphic, and contained a fine granular chromatin pattern with few nucleoli (Fig. 1a and c).

Colonies which contained cells with both SV40 and ad characteristics were round, sharply demarcated, and contained epithelial cells intermediate in size between the SV40 and ad cells which tended to pile up in the central portions of the colony. The nuclei were pleomorphic, larger than ad nuclei, and contained more coarse, clumped chromatin and nucleoli than ad-transformed cells; however, neither of these features were as prominent as in the SV40-transformed cells. Giant cells and cytoplasmic eosinophilic bodies were frequently present (Fig. 1b and d). Thus, the intermediate cell with mixed histological characteristics tended to have an ad morphology (though larger) and to form colonies morphologically similar to ad colonies. However, the nuclear characteristics were more similar to those of SV40-transformed cells; eosinophilic, cytoplasmic bodies, and giant cells, features characteristic of SV40 transformations, were present as well.

In Table 2, the morphological characteristics of the cell lines and clones under study are summarized. Three cell lines and their derivative clones were composed of cells with an intermediate morphology (ad 2<sup>+</sup> HK-4, ad 12<sup>+</sup> HK-1, ad 12<sup>+</sup> HK-4). One cell line and the clones derived therefrom contained cells which were typical of SV40-transformed cells (ad 12<sup>+</sup> HK-2). The remaining cell lines were composed of mixtures of cells, all of which were recovered in pure form in the clones isolated from these lines. The morphologic structure of SV40 or intermediate clones was constant whether they were derived from cell lines transformed by the ad 2<sup>+</sup> or ad 12<sup>+</sup> transcapiscent viruses. Two other cell lines transformed by the ad 2<sup>+</sup> transfer hybrid (ad 2<sup>+</sup> HK-1 and ad 2<sup>+</sup> HK-2) contained cells with a typical SV40 morphology as did cells transformed by the ad 2-SV40, ad

TABLE 2. Characteristics of clones derived from ad 2<sup>+</sup>t<sup>7</sup>- and ad 12<sup>+</sup>t<sup>7</sup>-transformed cell lines

Transformed cell line	Cytomorphology			Histomorphology					T antigen content <sup>a</sup>	
	SV40	Ad	Inter- mediate	SV40	Ad	Ad inter- mediate <sup>b</sup>	SV40 inter- mediate <sup>c</sup>	Total	SV40 T	Ad 12 T
Ad 2 <sup>+</sup> t <sup>7</sup> HK-3	+		+	4				4	+	-
Clone 1	+			8				8	+	-
Clone 3	+								+	-
Clone 8			+		3			3	+	- <sup>d</sup>
Clone 10			+		3			3	+	-
Clone 12			+						+	-
Clone 15	+								+	-
Ad 2 <sup>+</sup> t <sup>7</sup> HK-4			+		2	13		15	+	-
Clone 1			+		3	2		5	+	-
Clone 3			+		1	4	1	6	+	-
Clone 6			+	1	2	2		5	+	-
Clone 7			+			4		4	+	-
Clone 8			+		4		1	5	+	-
Ad 12 <sup>+</sup> t <sup>7</sup> HK-1			+		14			14	+	+
Clone 1			+		4			4	+	+
Clone 3			+		4			4	+	+
Ad 12 <sup>+</sup> t <sup>7</sup> HK-2	+			8				8	+	-
Clone 3	+			5				5	+	-
Clone 4	+			6				6	+	-
Ad 12 <sup>+</sup> t <sup>7</sup> HK-3	+	+			6			6	+	+
Clone 2	+			3				3	+	-
Clone 5	+			5				5	+	-
Clone 10		+			7			7	-	+
Clone 12		+			5			5	-	+
Ad 12 <sup>+</sup> t <sup>7</sup> HK-4			+		10			10	+	+
Clone 1			+		8			8	+	+
Clone 3			+		4			4	+	+
Ad 12 <sup>+</sup> t <sup>7</sup> HK-5	+		+		3			3	+	+
Clone 1	+				4 <sup>e</sup>			4	+	-
Clone 6	+				5 <sup>e</sup>			5	+	-
Clone 10			+		7			7	+	+

<sup>a</sup> Ad 7 or Ad 2 T antigens were not demonstrated in any of the cell lines or clones derived therefrom by the sera enumerated in the text.

<sup>b</sup> Predominantly Ad type tumors with intermediate features.

<sup>c</sup> Predominantly SV40 type tumors with intermediate features.

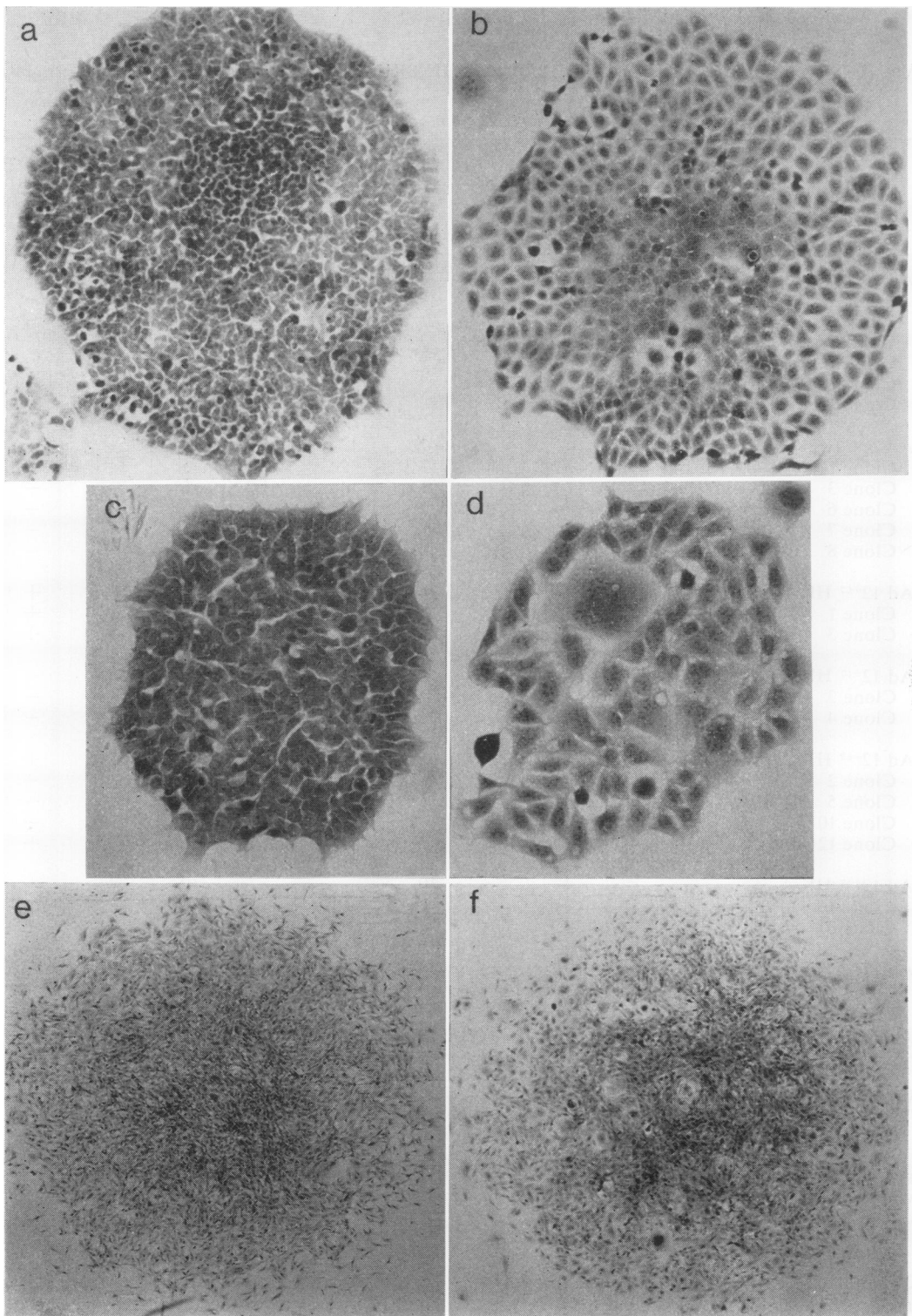
<sup>d</sup> Faint nuclear staining was occasionally observed in a very small proportion of cells in clones with an intermediate morphology derived from the ad 2<sup>+</sup>t<sup>7</sup> HK-3 and Ad 2<sup>+</sup>t<sup>7</sup> HK-4 cell lines when stained with the ad 12 hamster tumor sera pool. This staining was not considered unequivocal; it may have been due to the presence of a group reactive T antigen(s) (3, 24).

<sup>e</sup> Predominantly atypical ad type tumors.

3-SV40, and ad 7-SV40 passage hybrid viruses. These cell lines have been described in a previous publication (6).

Since the amount of calcium in the medium has a unique effect on the growth characteristics of adenovirus-transformed cell lines [rounding and clumping of the cells which ultimately detach from the glass occurs less frequently in medium

containing low concentrations (0.1 mM) of calcium (9)], the effect of calcium concentration on the morphology and growth characteristics of the hybrid-transformed cells was determined. Ad 2<sup>+</sup>t<sup>7</sup> HK-4 was maintained in the usual medium and in media containing 1.8 and 0.1 mM calcium for 10 passages (*see above*). No differences in morphology or growth characteristics



**FIG. 1.** Clones of *ad*-, *ad*-SV40-, and SV40-transformed cells; hematoxylin and eosin stain. (a) *Ad* 12<sup>+/+</sup> HK-3, clone 12; *ad* colony which is round, regular in outline, and composed of small, tightly packed epithelial cells.  $\times 120$ . (b) *Ad* 2<sup>+/+</sup> HK-3, clone 8; typical colony containing features of *ad*- and SV40-transformed cells; cells are epithelial but larger than *ad* cells; colonial morphology is similar to *ad* morphology, however.  $\times 120$ . (c) *Ad* 12<sup>+/+</sup> HK-3, clone 12; higher power of typical *ad* colony; nuclei fill practically entire cell and contain few nucleoli.  $\times 280$ . (d) *Ad* 2<sup>+/+</sup> HK-3, clone 8; higher power of intermediate colony; nuclei are larger than *ad* nuclei and contain coarser, clumped chromatin and more nucleoli; note presence of multinucleated giant cell.  $\times 280$ . (e) *Ad* 2<sup>+/+</sup> HK-3, clone 3; SV40 type colony with irregular margins composed of fibroblastic cells; note similarity to Fig. 1f.  $\times 30$ . (f) SV40-transformed hamster cell clone; SV40 colony with irregular margins composed of fibroblastic cells.  $\times 30$ .

were observed. Although the cells of clones 6 and 8, isolated from this cell line, did show a tendency to become round and grow in a serpentine fashion, these growth characteristics also were unaffected by maintenance and two passages in low calcium medium. However, ad 12-transformed hamster cells showed greater adherence to glass and less tendency to clump in media containing 0.1 mM calcium. Thus the calcium effect observed with ad-transformed cells was not demonstrated with the hybrid-transformed cells.

**Virus isolation.** Infectious SV40 or adenovirus were not isolated from any of the cell lines after overlay on AGMK and HEK indicator cultures and blind passage of dual culture extracts.

**Detection of T antigens.** Nearly all cells from the mass cultures with the exception of the ad 12<sup>+tr</sup> HK-3 cell line contained the SV40 T antigen (*see* Table 2). In the ad 12<sup>+tr</sup> HK-3 cell line, approximately 50% of cells contained the SV40 T antigen; clones 2 and 5 from this cell line, however, contained the SV40 T antigen in 95 to 100% of the cells, whereas clones 10 and 12 were devoid of SV40 T antigen. The former clones were SV40, whereas the latter were ad in morphology. The characteristic SV40 staining was granular, confined to the nucleus, and spared the nucleoli.

In cells transformed by the ad 12<sup>+tr</sup> transcapsidant virus, ad 12 T antigen was present in the cell lines and clones containing cells with an ad type or intermediate morphology and was absent in those with an SV40 type morphology. Cells stained by the ad 12 hamster tumor sera contained large and fine nuclear and occasionally cytoplasmic flecks. In addition, bright nuclear dots or balls were present in approximately 1 to 5% of cells, and diffuse, granular, nuclear staining which was more characteristic of late viral antigens (3, 7) was sometimes present in approximately 0.5 to 2% of cells (not necessarily those containing the nuclear dots or balls). In approximately 1 to 10% of cells, a single large cytoplasmic mass previously noted in cells transformed by ad 2 and 12 was seen (3, 20). These ad-staining patterns were present in clones derived from ad 12<sup>+tr</sup>-transformed cell lines which had either the typical adenovirus or the intermediate morphology.

Ad 7 and ad 2 T antigens could not be demonstrated in any of the cell lines with the sera utilized (*see* above). The serum pool derived from hamsters bearing transplanted tumors induced with the ad 1-SV40 hybrid virus gave typical SV40 staining despite repeated adsorptions with SV40-transformed cell extracts (*see* above). Difficulty in detecting the ad 7 T and ad 2 T antigens in these hybrid-transformed cell lines by FA tests has been noted previously (5, 6, 17, 30).

In summary, cells having an SV40 or intermediate type morphology contained SV40 T antigen. Cell lines derived from ad 12<sup>+tr</sup> hybrid virus transformations having an intermediate morphology contained ad 12-specific antigens as well. Cells with a typical ad morphology were stained only by the ad 12 tumor sera pool. Although no staining of ad 7 T and ad 2 T antigens could be demonstrated, evidence will be presented that all cells containing the SV40 T antigen also contain ad 7-specific genetic information, and cells from clones derived from ad 2<sup>+tr</sup> transformations which have an intermediate morphology contain ad 2-specific ribonucleic acid (RNA) as well (14).

**Transplantation and histomorphology.** Tumors were induced with all cell lines where transplantation was attempted. However, some of the lines were poorly tumorigenic in weanling animals and inoculation of newborns was necessitated. The latent period varied from 2 to 10 weeks and the duration and speed of tumor growth also varied considerably. In general, cells with an SV40 morphology transplanted most readily, whereas cells with an intermediate or ad morphology required longer latent periods, grew more slowly, and often had to be transplanted to newborn animals.

Grossly the tumors showed considerable variation in appearance, and lesions of several weeks duration invariably showed extensive areas of necrosis. However, most of the lesions could be categorized as firm, rubbery growths, or soft, white, friable masses. Histology confirmed the SV40 pattern in the former and ad type morphology in the latter lesions.

Histologically (Table 2) most of the tumors appeared to be characteristic SV40 or ad type lesions and showed the typical features described previously (1). Clones with an SV40 or ad type morphology gave rise to tumors with typical SV40 or ad histologies, respectively (Fig. 2a and b). The only exceptions were ad 12<sup>+tr</sup> HK-5, clones 1 and 6, which produced tumors with ad characteristics. However, the predominant cell type in these tumors was somewhat atypical in that it was larger and contained more abundant, distinct cytoplasm. Areas of this type of tumor growth were also seen in one of the ad 12 HK-5 uncloned tumors (Fig. 2c).

Most clones with an intermediate morphology gave rise to typical ad tumors. The sole exception was ad 2<sup>+tr</sup> HK-4 in which many tumors induced by the mass culture and its derived clones showed some unusual features, which tended to give these lesions an intermediate character. Most of the tumors were basically ad type tumors in which focal areas assumed a rough spindle cell configu-

ration characteristic of SV40 lesions. However, the nuclei of these cells remained characteristic of the ad pattern, featuring delicate punctate chromatin (Fig. 2d). In some tumors there was

a frank mosaic pattern containing areas of adjacent ad and SV40 type growth and areas of intermediate type with the mixed features described above (Fig. 2e). Two of the tumors were

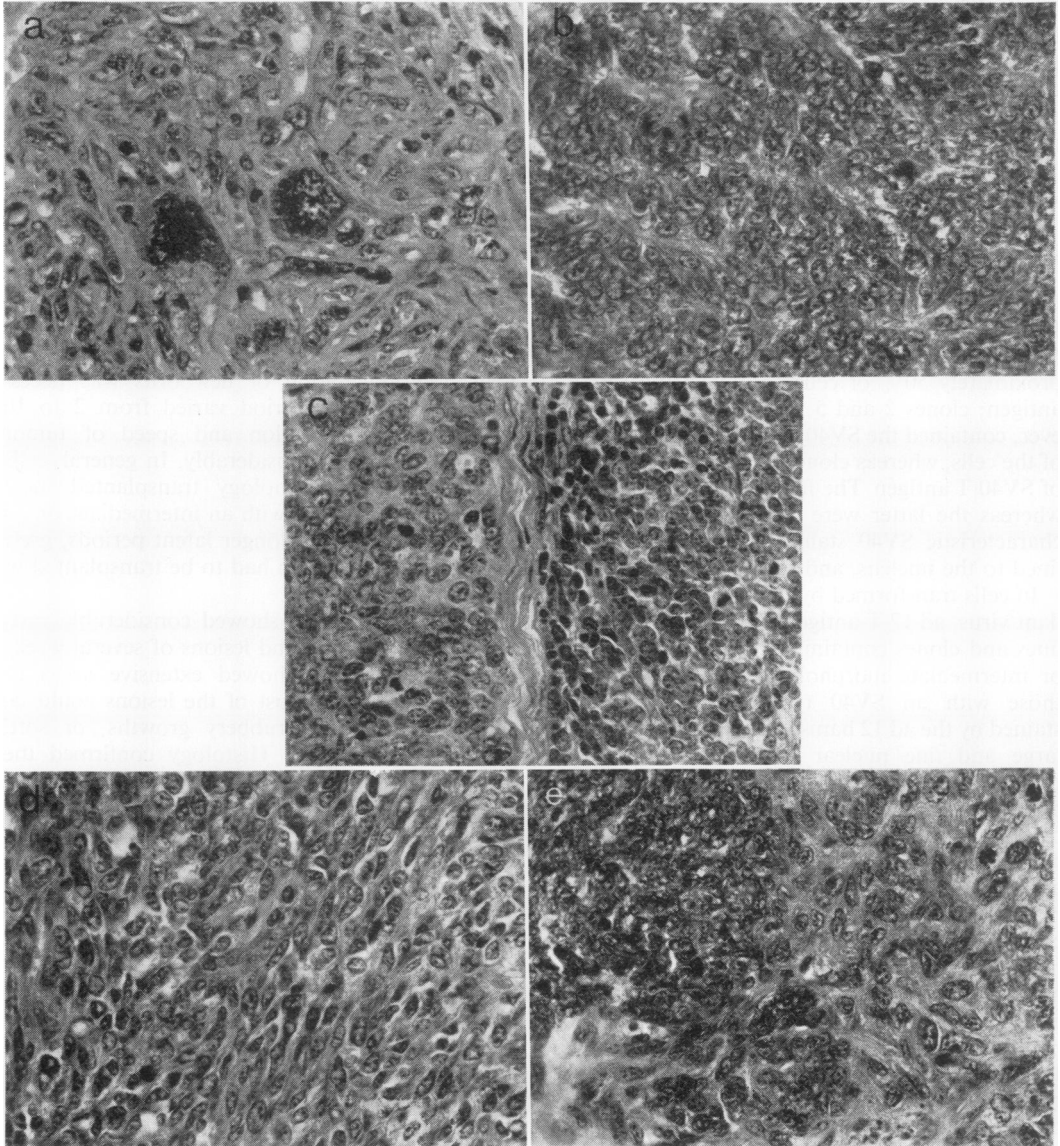


FIG. 2. Tumors induced with ad  $2^{+/-}$ - and ad  $12^{+/-}$ -transformed cells. (a) Typical SV40 type sarcoma induced by ad  $12^{+/-}$  HK-2 cells; note characteristic giant cells and cells with vesicular nuclei with single central nucleolus.  $\times 450$ . (b) Typical small cell ad type tumor induced by ad  $12^{+/-}$  HK-3 clone 10 cells; note nuclei with delicate punctate chromatin and palisading of tumor cells around stromal projections.  $\times 460$ . (c) Tumor induced by ad  $12^{+/-}$  HK-5 cells; note presence of two adjacent tumor nodules: one is typical ad tumor similar to Fig. 2b, the other is composed of slightly larger cells with more abundant, discrete, cytoplasm; the nuclei, although slightly larger, are basically similar.  $\times 450$ . (d) Tumor induced by ad  $2^{+/-}$  HK-4 clone 1 cells; this is a spindle cell sarcoma with cells in wavy and whorled patterns; nuclei are basically ad type, however.  $\times 570$ . (e) Same tumor as Fig. 2d; area of dimorphic growth with adjacent patterns resembling ad and SV40 type tumor.  $\times 450$ .

basically SV40 type in which there were focal areas of uniform, round cells suggestive of an ad tumor.

Some of the ad type tumors contained large giant cells which resembled those typical of SV40 lesions. In some clones the presence of these giant cells correlated with an intermediate type of cytomorphology in tissue culture. However, the correlation was not absolute, and many cytomorphologically intermediate clones contained few, if any, giant cells.

### DISCUSSION

From an analysis of the different clones isolated from the ad 2<sup>+</sup> and ad 12<sup>+</sup>-transformed hamster cell lines, it is evident that at least three different transformation events occurred. With nucleic acid hybridization techniques the evidence for the presence of different virus-specific RNA's, in each of these types of clones, together with a discussion of the probable mechanism of transformation, will be presented in the accompanying paper (14). The clones with a typical ad morphology which contained only ad 12 T antigen and ad 12-specific RNA were presumably derived from transformations by the ad 12 virions in the mixed viral population. The remaining clones contained both ad and SV40 genetic information. Those clones with an SV40 type morphology contained ad 7 and SV40 genetic information and probably represent transformation by the defective transcapsidant particle, whereas clones which were intermediate in morphology contained either ad 2 or ad 12 genes, depending on the transcapsidant virus used, together with ad 7 and SV40 genetic information; the latter clones presumably arose from dual infection of cells by the adenovirions and transcapsidant particles which comprise these viral populations (14).

From a study of the cytology and tumors derived from the clonal lines, the original transformed cell lines may be grouped into 2 categories, those homogeneous and those heterogeneous with respect to cell type. The homogeneous cell lines (ad 12<sup>+</sup> HK-2, ad 12<sup>+</sup> HK-1, ad 12<sup>+</sup> HK-4, and ad 2<sup>+</sup> HK-4) gave rise to clones which were alike in morphology and which resembled the parent mass culture. Ad 12<sup>+</sup> HK-2 cells produced SV40 type tumors exclusively, as did its derivative clones. Thus, the SV40 component of the ad 7-SV40 complex in this cell line appears to be dominant during cell growth *in vivo*, which parallels the cytomorphology *in vitro*. The ad 12<sup>+</sup> HK-1 and HK-4 lines which contained cells which were intermediate in morphology gave rise to ad type tumors upon transplantation. It appears, therefore, that tissue

culture cytology is more sensitive than tumor histology in the detection of intermediate features in the cell lines. The ad 2<sup>+</sup> HK-4 cell line, however, which was also intermediate in cytology, gave rise to tumors which exhibited the expression of both ad and SV40 genomes. A wide spectrum of morphological variation was present in tumors produced by individual clones of this cell line (clones 3, 6, and 8). These results, as well as the findings with tumors induced by cells cloned from SV40-transformed WHK cells (4), imply that the progeny of single transformed cells can undergo a significant degree of morphological variation. It is possible that "morphological modulation," in which the morphology of tumor cells can shift through a limited spectrum, may be a factor determining the histology of these tumors, and may have accounted for the dimorphic picture encountered at least in these lesions. Certainly the presence of intermediate features in the ad 2<sup>+</sup> HK-4 tumors would imply that the morphological expression of the ad and SV40 genomes were both being expressed at the single cell level *in vivo* and *in vitro*.

The intermediate histological features of the ad 2<sup>+</sup> HK-4 lesions are similar to tumors induced by other cell lines transformed with the ad 2-SV40, ad 3-SV40, and ad 7-SV40 passage hybrids, and the ad 2<sup>+</sup> transcapsidant hybrid viruses described previously (11). That report also described focal changes in one ad type tumor involving the presence of enlarged vesicular nuclei with coarsely clumped chromatin (reference 11, Fig. 15). This was also seen in a number of the ad type tumors in this study and appeared to be more associated with tumors induced by cells having an intermediate cytology. However, as with the presence of giant cells, this correlation was variable and inconstant.

The three remaining lines in the present study, ad 2<sup>+</sup> HK-3, ad 12<sup>+</sup> HK-3, and ad 12<sup>+</sup> HK-5, were all heterogeneous, being composed of mixtures of different types of transformed cells. Each clone derived from these cell lines was composed of a homogeneous population of cells, and the tumors derived therefrom were similar to the tumors derived from the cell lines described above. In addition, two clones derived from the ad 12<sup>+</sup> HK-3 cell lines (clones 10 and 12) exhibited typical ad morphology both *in vitro* and *in vivo*. A further type of phenotypic expression was exemplified by clones 1 and 6 derived from the ad 12<sup>+</sup> HK-5 cell line. These clones were composed of cells with an SV40 type cytology; however, the tumors derived from these clones were basically ad in morphology. The reason(s) for this discrepancy is not apparent; it may be due to differences in the amount of ad 7 genetic



information present in these lines, relative to other lines containing these genomes, or in its degree of expression. This finding, however, indicates that ad morphological determinants may be present in the ad 7-SV40 complex.

The studies presented indicate that the morphology of a cell is determined by the type and amount of viral genetic material present. This provides additional evidence that the viral genome directs the morphology of the transformed cell. Previous studies with the Rous sarcoma virus (34) and ad-transformed BHK cells (33) also indicate that the viral genome is the main determinant of the phenotype of the transformed cell. The present studies indicate that when mixtures of viral genomes are present their interaction, as well as conditions of cellular growth, may influence the phenotypic expression of the various viral genomes.

The data presented confirm, extend, and explain many of the findings reported by others and they emphasize that histological examination of tumors induced by mass cultures of transformed cells or mixed virus populations is often misleading. Thus tumors induced with the ad 2<sup>+17</sup> HK-3 and ad 12<sup>+17</sup> HK-3 mass cultures were typically SV40 and ad in histology, respectively; however, clones with intermediate morphology could be isolated from the former whereas SV40 type clones were isolated from the latter cell lines. It is most likely that growth of these cell lines *in vivo* selected against one of the components in the original cell mixture.

The fact that cells with the ad 7-SV40 genomes may give rise to tumors with an SV40 or ad morphology helps explain the diversity of findings with tumors induced with the ad 7-SV40 passage hybrid virus or transplantation of cells transformed with this virus population, since tumors with SV40 morphology (5, 12, 17), ad morphology (22), or mixed morphological characteristics (10, 22, 26) have resulted. Mixtures of cells, representing several transformation events, dual infection of a cell by both ad 7 virions and the hybrid particle, or variable expression of the viral genomes present, may also have influenced the tumor histology. The same multiplicity of findings exists with other hybrid populations. Thus, ad 12-SV40 hybrid-transformed cells were reported to have an ad morphology in some studies (17, 32). Ad 12-SV40-induced tumor cells, however, have been reported to contain either ad 12 T, SV40 T, or both T antigens (17). The clones *resulting* from these tumor cells probably *resulted* from separate transformation events by the ad 12 virions, the transcapsidant particle, or both particles in the hybrid population. In another study, tumor cells

derived from tumors induced with the ad 12-SV40 transcapsidant virus were fibroblastic and contained no ad 12 T antigen (13). This tumor probably arose from transformation with the transcapsidant particle and presumably contains the ad 7 and SV40 genomes but no ad 12 genetic information. In the majority of studies, mixed or dimorphic tumors resulted from inoculation of animals with transcapsidant viruses (22, 26, 35, 36). Such tumors probably represent several oncogenic events by the various particles in the hybrid population and are analogous to some of the mass culture lines in the present and past studies (11). Cloning, therefore, is an essential procedure in the study of transformation affected by mixtures of oncogenic viruses of which the defective transcapsidant populations are a prime example.

#### ACKNOWLEDGMENT

This investigation was supported by Public Health Service grant no. CA 10126-02 from the National Cancer Institute, and by grant E-550 from the American Cancer Society.

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