AN ANALYSIS OF SV40-INDUCED TRANSFORMATION OF HAMSTER KIDNEY TISSUE IN VITRO, II. STUDIES OF THREE CLONES DERIVED FROM A CONTINUOUS LINE OF TRANSFORMED CELLS

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In an earlier report on the characteristics of transformation of hamster kidney cell cultures by SV40 virus, a continuous cell line (THK-1) of transformed cells was described.[1] This cell line showed two unusual characteristics, namely, the continued presence of SV40 in a form or amount difficult to detect, and induction of transplant tumors containing both carcinomatous and sarcomatous elements. The interpretation of both of these findings depends on whether the cells are homogeneous in these respects, or whether the findings are the result of mixtures of cell types which are heterogeneous with respect to carriage of virus and type of growth *in vivo*. To clarify these points, clones were established, in the presence of antiserum, from the THK-1 cell line, and cell lines established from them. In this report, a study of three such clones and the tumors produced in hamsters by transplantation of the cloned cells will be presented. These three clones have all been found to yield infectious virus when the clone cells are implanted on Cercopithecus kidney monolayer cultures, providing further evidence for the integration of the SV40 viral genome in a subinfectious state in transformed hamster kidney cells.

Materials and Methods.—Cloning technique: Cells were cloned from the continuous transformed line THK-1, without the benefit of feeder cells; the cloning procedure of Puck[2] employing small cylinders was employed. Cells of the 29th passage were dispersed by trypsinization; a sample was counted in a hemocytometer, and the cells were observed to be monodisperse. One hundred cells in 5 ml of growth medium were dispensed into each of four plastic Petri dishes 60 mm in diameter. The growth medium consisted of NCTC-109 with 15% unheated fetal calf serum, 0.5% heated hyperimmune rabbit anti-SV40 serum (neutralizing antibody titer > 2560), and antibiotics. The cultures were incubated at 37° C in 5% CO_2-humidified atmosphere. One dish was examined on the 3rd day and tiny clones containing 2–4 cells were present. The remaining cultures were left undisturbed until the 7th day. At this time there was an average of 35 colonies per plate. Three small, well-isolated colonies, two (#4-2 and #4-2) epithelioid and one (3-3) of more fibroblastic appearance, were considered to represent clones and were selected for passage. A sterile penicillin assay cylinder was rimmed with sterile silicone stopcock grease (Dow-Corning) and placed over each clone; the cells were removed by trypsinization and transferred to separate culture dishes. The cells grew rapidly and formed confluent monolayers in 6–10 days. The growth medium containing antibody was used for 6 passages; thereafter, the same medium was used, but without the rabbit antiserum.

Virus isolation from clone cells: The four methods used for determination of virus in transformed cultures have been described.[1] In the overlay method, the clonal lines so rapidly overgrew the monkey kidney (AGMK) cells that the latter cells could not be observed for more than 14–17 days in most instances. Therefore, blind passages were made, generally at the time of complete overgrowth, by disrupting cells by freeze-thawing the culture tubes 2–3 times or extracting with chloroform,[1] pooling the contents of replicate cultures, and inoculating 0.1 ml of the disrupted cell suspension into three AGMK culture tubes. These were observed for 30–40 days for occurrence of characteristic SV40 cytopathic effects (CPE).

Transplantation: Procedures used for transplantation of tissue culture cells into weanling hamsters and for viral and histologic studies have been described previously.[1, 3] Tumors which

appeared purely sarcomatous by histological examination were resectioned at four different levels of the tumor in order to verify the absence of epithelial elements.

Chromosome studies: Cytogenetic studies were performed by techniques previously described.[4]

Results.—Growth characteristics in tissue culture: The three clonal lines have been carried without difficulty through 40 serial passages in tissue culture over a period of 196 days after cloning. The cells grow very rapidly and produce marked acidification of the medium. Through most of the passage series, clone 3-3 was composed of a spectrum of cell types ranging from thin fibroblastic cells to short triangular-shaped fibroblasts with a few cells of epithelioid appearance. Few giant cells were present. The cells grew in strands and whorls in a random, crisscross fashion and piled up to form nodular growth centers (Fig. 1). These cells loosened individually when exposed to trypsin. At the 37th passage, stained preparations showed predominantly polygonal cells. Clones 4-1 and 4-2 are composed of polygonal, epithelioid cells which grow into thick, multilayered sheets. Many more giant cells are present than in clone 3-3 (Fig. 1). When treated with trypsin, these clones loosen as sheets of cells. Some cells from all clones contain eosinophilic cytoplasmic masses of various sizes; similar structures have been seen in uninoculated primary cultures of weanling hamster kidney cells.

Isolation of virus from clone cells: The virus recovery data on the three clonal lines are summarized in Table 1. No virus was detected in the supernatant culture fluids at any time. With one exception, extracts of concentrated cell suspensions containing 2–4 $\times$ 10^6 cells/ml were free of detectable virus; an extract of 6th-passage clone 3-3 cells contained a trace amount of virus. However, virus was readily recovered from all three clonal lines by the overlay procedure (Table 1); in almost all instances blind passage was necessary for virus detection. Control AGMK cultures, extracted and passed in a similar fashion with each overlay test, have never yielded SV40.

Transplantation of clone cells to hamsters: 2–3 $\times$ 10^6 cells of each clone at the 5th passage level were inoculated subcutaneously into seven weanling hamsters. Tiny subcutaneous nodules developed at the site of inoculation in all animals at 4–6 weeks and grew to a size of 1 cm (mean tumor diameter) within 2–3 weeks. Tumors arising from the cells from clone 3-3 grew much faster than those arising from the other two clones. Five tumors from clone 3-3 and four each from clones 4-1 and 4-2 were examined histologically and tested for virus content. SV40 was recovered from two of the 3-3 tumors, but no virus was detected in extracts of the 4-1 and 4-2 tumors (Table 1). It is possible that these tumors would have yielded virus if tested by the overlay method.[5, 6]

Grossly, the tumors resembled those arising from transplantation of THK-1 cells.[1] Often the entire central portion of the tumor was necrotic. The tumors remained localized and metastases were never observed grossly in six tumorous animals. Histologically, the tumors from the cells from clone 3-3 were all sarcomas in varying stages of malignancy, closely resembling primary tumors in virus-inoculated hamsters as well as the purely sarcomatous tumors often produced by transplants of the parent THK-1 cell line. (See Fig. 2(*B*) in ref. 1.) Several tumors had areas which were very cellular and anaplastic as well as areas of frank fibrosarcoma. Some tumors arising from cells of clones 4-1 and 4-2 were purely sarcomatous, but three tumors from clone 4-1 and one from clone 4-2 had many areas

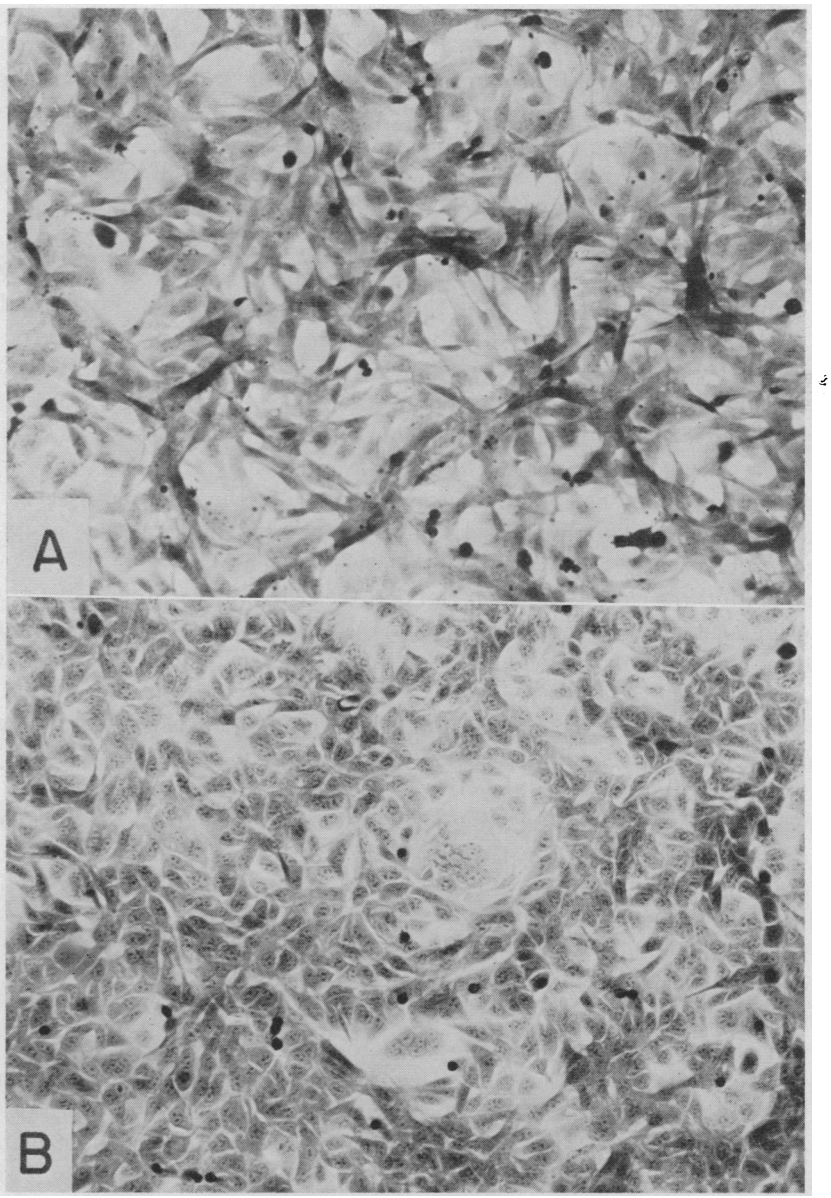

FIG. 1.—(*A*) Clone 3-3, 9th passage. Note cellular pleomorphism but predominantly fibroblastic morphology. H & E, ×143. (*B*) Clone 4-2, 9th passage. Note epithelioid morphology of cells, the multinucleated giant cells, and the numerous mitotic figures. H & E, ×143.

of epithelial differentiation into tubular structures, as well as sarcomatous areas (Fig. 2). These mixed tumors were identical to the carcinosarcomas frequently produced by the THK-1 cells. (See Fig. 2(*A*) in ref. 1.) All tumors from the clone cells had many multinucleated giant cells. Some tumors of both mixed and sarcomatous histology had invaded adjacent muscle; several blood vessels in both types of tumor also contained tumor cells within their lumen.

Chromosome studies: Prior to cloning, the THK-1 cell line showed marked variability in chromosomal number and morphology. A distribution analysis of the chromosome numbers of the clones was made at the 3rd, 10th, and 35th passage levels (Fig. 3). At the third passage after cloning, each of the three clones showed a different predominating modal chromosome number.

At the tenth passage, clone 3-3 still showed a marked predominance of the same modal chromosome number, with a narrow distribution about the mode. Clone 4-2 also showed its original modal number, but the distribution about the mode had a greater spread than in the earlier passage, and the frequency of cells with the modal number was lower than at the third passage. Clone 4-1 also showed an increase in the width of the distribution about the mode and a considerable reduction in the frequency of modal cells.

At the 35th passage, all three clones showed marked variation in chromosome numbers. Clone 3-3 had a new modal number with relatively low frequency of modal cells. Clone 4-1 appeared to have become bimodal, neither of the modes having the same chromosome number as the mode of the earlier passages. Clone 4-2 had a chromosome-number distribution similar in shape to that of its earlier passages, but an entirely new modal number was present.

Thus, in all three clones, during serial passages there was a progressive alteration of the original stem-like karyotype (presumably that of the cell from which the clone derived). Detailed karyotype analyses are being carried out to determine whether there is any tendency toward stabilization of one or a few karyotypes or whether the clones will ultimately come to resemble the parent line. These studies will also determine whether the modal cells constitute a true stem line, or are actually karyotypically heterogenous.

Discussion: From the preceding experiments, it is clear that transformed hamster kidney cells, cloned in the presence of high-titered antiserum, yield infectious virus material. There seems to be no question that the clones could have been infected with extracellular virus at the time of cloning because the culture fluids had been devoid of infectious virus for 26 passages; in addition, the amount of antiserum used should have been far more than adequate to neutralize any trace amounts that may have been present. Although only three clones were studied (and on a purely statistical basis this would only give assurance (at the 0.05 level) that at least 37 per cent of the THK-1 cells would yield virus), it is quite probable that all the transformed cells have this potential. Thus, clones of both of the cell types present in the original culture gave comparable results. Also, the similarity of these results to those of Sabin and Koch[7] supports this contention; their investigations indicated that a high proportion of hamster tumor cells induced by SV40 *in vivo* contain subinfectious SV40 viral genome.

Although it is clear that the viral genome is present in a high proportion of the transformed cells, with the exception of one test (24th passage of THK-1[1]), they contained less than one $TCID_{50}$ of extractable virus per 10^6 cells. Whether the infectivity recovered by the overlay method represents whole virus or infectious DNA[8] being liberated by cells, either as a rare burst or as an extremely slow trickle from a high proportion of cells, is not known. While it is conceivable that the viral DNA is being replicated without concomitant replication of viral protein components, there is strong evidence that the viral genome is efficiently directing synthesis

TABLE 1

RECOVERY OF SV40 FROM THREE CLONAL LINES OF TRANSFORMED HAMSTER KIDNEY CELLS

Clone	Expt.	Passage	Test for virus in supernatant	Test for virus in disrupted cells	Overlay Method: No. cells overlaid (log_{10})	Overlay Method: Result	Overlay Method: Length of observation (days)	Blind passage result	Virus in tumors
3-3	1-8	5							2/5[d]
	1-15	6	−	+[a]					
	1-31	9			5.8	−	17	+	
		9			5.2	−	17	+	
		9			4.2	−	17	+	
	3-14	18			5.6	−	11	−	
		18			4.6	−	11	+	
	3-27	21	−	−	5.6	−	14	−	
		21			4.6	−	34		
	4-18	28		−					
	5-23	35			5.2	−	18	+	
					4.2	−	18	+	
					3.2	−	18	+	
	1-8	5							0/4
4-1	1-15	6	−	−					
	1-31	9			5.8	−	17	+	
		9			5.2	+(1/3)[b]	17	+[c]	
		9			4.2	−	17	+	
	3-14	18			5.6	−	11	+	
		18			4.6	−	11	+	
	3-27	21	−	−	5.6	−	14	+	
		21			4.6	+(1/3)	34		
	1-8	5							0/4
	1-15	6	−	−					
4-2	1-31	9			5.8	−	17	+	
		9			5.2	−	17	+	
		9			4.2	−	17	+	
	3-14	18			5.6	−	11	−	
		18			4.6	−	11	+	
	3-27	21	−	−	5.6	−	14	+	
		21			4.6	+(3/3)			

[a] Positive on blind passage.
[b] No. tubes positive/no. tubes inoculated.
[c] The two negative tubes were blind passed.
[d] No. tumors positive/no. tumors tested.

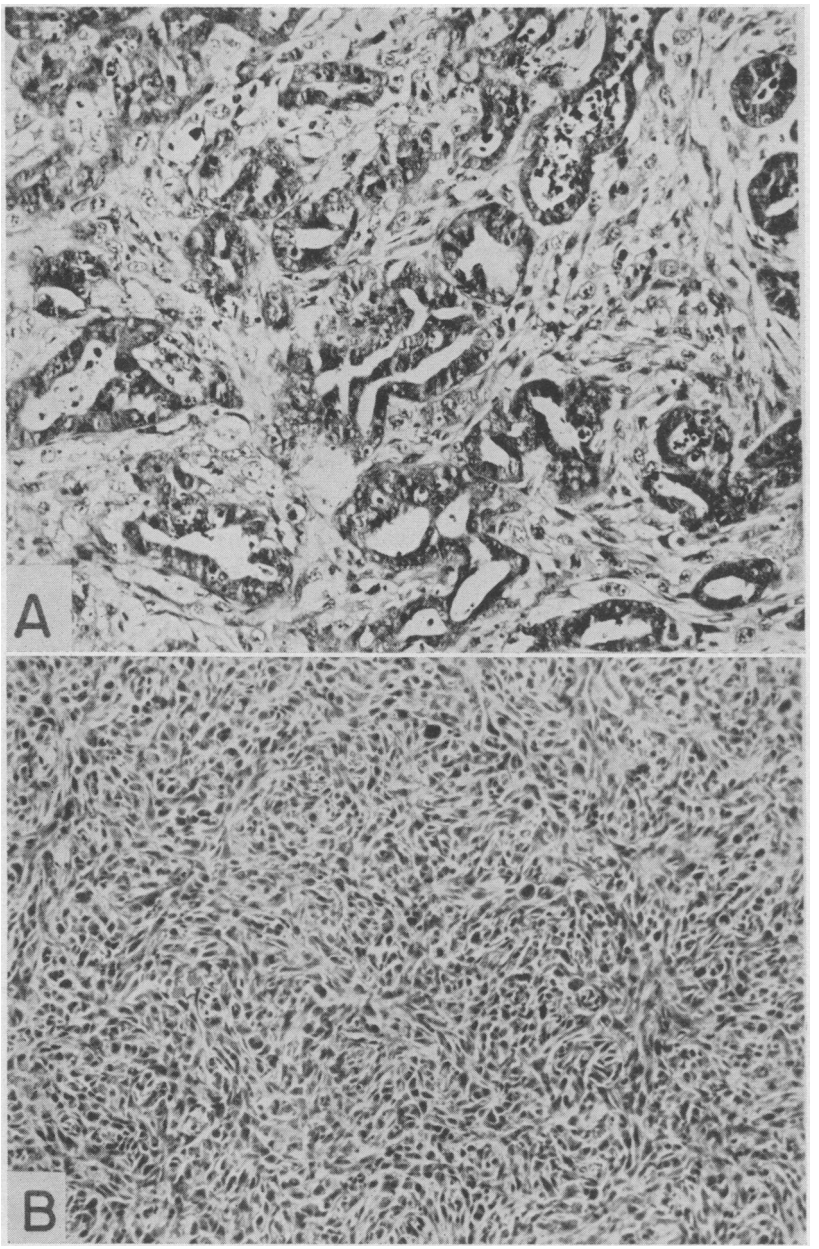

FIG. 2.—(*A*) Mixed tumor arising from transplantation of cells from clone 4-2, showing area of epithelial differentiation into tubular structures. H &E, ×143. (*B*) Well-differentiated fibrosarcoma arising from transplantation of cells from clone 4-2. Several multinucleated giant cells are present. H & E, ×143.

of at least one virus-specific substance, namely, the specific complement-fixing antigen present in tumors and transformed cells of various species origin.[9] This antigen may be identical to the transplantation antigen demonstrated in SV40 hamster tumors.[10–12] Whether the genome is associated with host-cell chromosomes, as in bacterial lysogeny, is not known.

In similar studies of clones of human cells transformed by SV40, Pontén *et al.*[13] found virus in a high proportion of clones. Their results are difficult to compare with ours, however, since the cloning was not done in the presence of antiserum, and the overlay procedure was not used.[5, 6]

Transplantation of the clonal lines indicated that a single epithelioid transformed cell is capable of differentiating into cells with either fibroblastic morphology or epithelial cells capable of forming tubular structures. The fibroblastic clone 3-3 gave rise only to sarcomatous tumors.

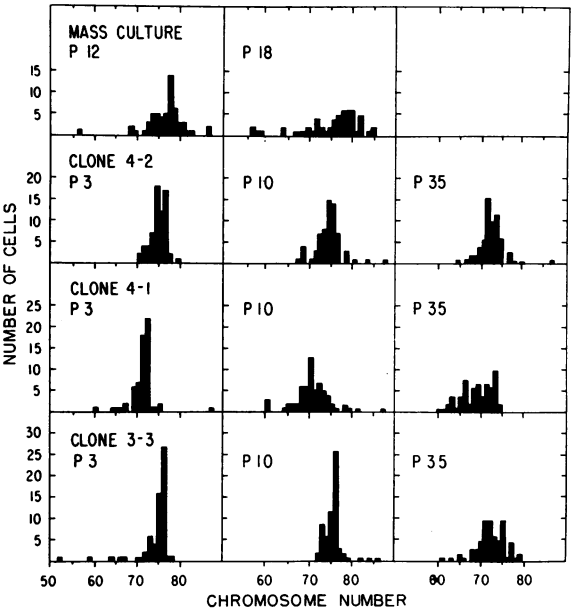

FIG. 3.—Chromosome-number distributions of cells from THK-1 line and from three clones derived by single-cell isolation. Passage numbers are indicated.

The induction of mixed tumors by clonal lines lends support to the concept that the initially transformed cell may be of a single-cell type. This may be primordial mesenchymal cell which serves as the precursor of both the fibroblastic and epithelial cells. It is of interest that the avian renal nephroblastoma, a mixed tumor composed of epithelial and fibrosarcomatous elements resulting from infection with the avian leukosis virus, develops from primitive nephrogenic mesenchyme residual in the postembryonic kidney.[14, 15] The hamster kidney is incompletely developed at birth, and nests of these mesenchymal cells are interspersed between the tubules.[16] Although transformation might occur in these elements in embryonic kidney monolayers, it is doubtful that such cells would be present in the kidneys of 7-week-old hamsters.

Since both tubular epithelium and stromal tissue develop from the primitive, mesenchymal cells that comprise the metanephric anlarge,[17] an alternative hypothesis would be that infection of either tubular or stromal elements might result in a disturbance of differentiating mechanisms with subsequent morphologic variability.

The cytogenetic findings are of interest when compared to the histopathology of these tumors. While all three clones ultimately underwent deterioration of the original stem line, the rate at which this occurred differed. Clone 3-3 appeared to maintain its modal chromosome number relatively well for at least 10 passages. Clone 4-1 showed rapid deterioration beginning with 3 passages, while clone 4-2 was intermediate. Thus, at the time of animal inoculation (5th passage), clone 3-3 still had a strongly predominating modal chromosome number while clones 4-1 and 4-2 had begun to deteriorate karyotypically. It is conceivable then that the number of cell types produced on transplantation was related to the degree of instability of the karyotype.

The efficacy of the cloning technique used here for the isolation of clones derived from single cells is always subject to some question. Although no clumps of cells were seen in the examination made at the time of plating, the possibility that a few clumps were present cannot be excluded. The relative homogeneity of chromosome number in each clone when first examined speaks for their single-cell origin. The interpretations of the virologic data presented here would not be affected seriously if the "clones" had originated from small clumps. Concerning the induction of mixed tumors by clonal lines, the clones (4-1 and 4-2) which gave rise to such tumors were homogeneous with respect to cell type, and thus would not appear to be derived from mixtures of epithelioid and fibroblastic parent cells. Clone 3-3, which showed most variability in cell morphology in the early passages and would be most suspect in being derived from a clump of cells, gave rise to pure sarcomata.

Summary.—Three clonal lines, two of epithelioid and one of fibroblastic morphology, were established in the presence of SV40 antiserum from a continuous cell line of hamster kidney cells transformed by SV40. Virus was consistently recovered from all three clones only when they were planted directly on Cercopithecus kidney cultures. This suggests that the SV40 genome is integrated in the genetic apparatus of a high proportion of transformed hamster kidney cells.

Mixed tumors (carcinosarcomas) arose after transplantation of the epithelioid clones, indicating that a transformed cell may differentiate into cells with either an epithelial or fibroblastic morphology.

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