JOURNAL OF VIROLOGY, June 1976, p. 1106-1119
Vol. 18, No. 3

Printed in U.S.A.

Characterization of Simian Cells Transformed by Temperature-Sensitive Mutants of Simian Virus 40

CHRISTINE A. NOONAN, JOAN S. BRUGGE,[1] AND JANET S. BUTEL*
Department of Virology and Epidemiology, Baylor College of Medicine Houston, Texas 77030

Received for publication 24 December 1975

Seven lines derived from primary African green monkey kidney cells, which had survived lytic infection by wild-type simian virus 40 (SV40) or temperature-sensitive mutants belonging to the A and B complementation groups, were established. These cultures synthesize SV40 tumor (T) antigen constitutively and have been passaged more than 60 times in vitro. The cells released small amounts of virus even at high passage levels but eventually became negative for the spontaneous release of virus. Virus rescued from such "nonproducer" cells by the transfection technique exhibited the growth properties of the original inoculum virus. Four of the cell lines were tested for the presence of altered growth patterns commonly associated with SV40-induced transformation. Although each of the cell lines was greater than 99% positive for T antigen, none of the cultures could be distinguished from primary or stable lines of normal simian cells on the basis of morphology, saturation density in high or low serum concentrations, colony formation on plastic or in soft agar, hexose transport, or concanavalin A agglutinability. However, the cells could be distinguished from the parental green monkey kidney cells by a prolonged life span, the presence of T antigen, a resistance to the replication of superinfecting SV40 virus or SV40 viral DNA, and, with three of the four lines, an ability to complement the growth of human adenovirus type 7. These properties were expressed independent of the temperature of incubation. These results indicate that the presence of an immunologically reactive SV40 T antigen is not sufficient to ensure induction of phenotypic transformation and suggest that a specific interaction between viral and cellular genes and/or gene products may be a necessary requirement.

Infection of cells by simian virus 40 (SV40) can result in a productive or abortive infection depending upon the species of origin and/or the physiological state of the host cell used. Cells can be divided into three categories on the basis of their ability to support the replication of this virus. Cultures of African green monkey kidney (GMK) cells are fully permissive for SV40 replication; viral DNA and capsid proteins are synthesized, large quantities of fully infectious progeny virions are produced, and lysis and death of the simian cell results (41). In semipermissive cells (e.g., human), low levels of progeny virions are produced from a small percentage of cells. Nonpermissive cells (e.g., hamster, mouse) support only a partial expression of the viral genome; viral DNA is not replicated and progeny virions are not produced (41).

Both nonpermissive and semipermissive cells can be transformed by SV40 at frequencies of approximately 10 and 0.03%, respectively. Such cells display many, if not all, of the classical parameters associated with the transformed state (8). Studies with various species of semi- and nonpermissive cells transformed by the group A temperature-sensitive (ts) mutants of SV40 have suggested that the SV40 gene A product is involved in the maintenance of the transformed state (3, 16, 21, 25, 39).

Although most simian cells are killed after exposure to SV40, in rare instances, usually after some manipulation of the experimental conditions, clones of cells have been isolated that have survived the lytic infection (10, 20, 34, 42). Serial passage of such clones has resulted in the establishment of cultures, the cells of which are positive for the constitutive expression of the intranuclear tumor (T) antigen, yet fail to produce viral capsid (V) antigen or infectious virions. To date these cultures have been referred to as "transformed." However, most of the cell lines have not been tested for the presence of those growth alterations commonly associated with viral transformation. In addition, the role of the gene A product (if any) in the maintenance of the transformed

[1] Present address: Department of Pathology, University of Colorado Medical Center, Denver, Colo. 80220.

phenotype of simian cells has not been assessed.

In the present study, simian cells that had survived a lytic infection by wild-type (WT) virus or a series of ts mutants of SV40 and that synthesize T antigen constitutively were selected and screened for the presence of several specific markers of transformation. The putative role of the gene A protein in the maintenance of such phenotypic markers was also examined. The results of these experiments indicate that none of the transformed simian cells tested could be distinguished from primary or established lines of normal monkey cells by the parameters of saturation density in high and low serum concentrations, colony formation on plastic and in soft agar, transport of 2-deoxy-D-glucose (deoxyglucose), and agglutination by concanavalin A (ConA). However, these cells were distinct from the parental cells in that they exhibited, in addition to a prolonged life span and the presence of the SV40 T antigen, a resistance to superinfection by SV40 virus or viral DNA and, with one exception, the ability to enhance the growth of human adenovirus type 7.

MATERIALS AND METHODS

Viruses. The SV40 WT virus used was the Baylor reference strain, which had been plaque purified three times at 40.5 C. Also incorporated into this study were several group B mutants, which had been isolated in this laboratory after mutagenesis of WT virus with nitrous acid or nitrosoguanidine (C. A. Noonan and J. S. Butel, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, V219, p. 237). Mutant tsA28 was graciously provided by P. Tegtmeyer. SV40 WT and mutant stocks were prepared at 33 C in TC-7 cells (obtained from J. Robb) as described previously (3). The mutant viruses used in this study had been passed one to two times in TC-7 cells at a multiplicity of infection (MOI) of 0.01 PFU/ml. Assays for SV40 infectivity were performed at 33 and 40.5 C in TC-7 cells (33).

Adenovirus type 7 (Hu), a human isolate provided by M. Benyesh-Melnick, had been passed five times in human embryonic kidney cells as described (4). Accurate temperature control was obtained through the use of constant-temperature water baths (Blue M Electric Co., Blue Island, Ill.) and thermostatically controlled CO_2 incubators (Wedco, Inc., Silver Spring, Md.).

Cells. The following primary and stable lines of normal cells were used during the course of this investigation: BALB-3T3 cells (provided by W. Parks) derived from BALB/c mouse embryo cells and selected for a high degree of contact inhibition (40); primary human embryonic kidney cells purchased from Flow Laboratories and passaged two or three times; primary cultures of GMK cells used at the first or second passage level; and TC-7 cells, a stable clonal line of GMK cells derived fron the CV-1 line.

In addition, several lines of transformed simian cells were used: BSC-1-S cells, a clonal derivative of the BSC-1 line, which had been transformed in vitro by SV40 WT virus (20), supplied by N. Goldblum; T-22 cells, a line of GMK cells transformed by a heavily irradiated stock of the T fraction of SV40 (34), made available by H. Shimojo; GMK/PARA-7-1, primary GMK cells transformed in vitro by PARA-adenovirus 7 (J. S. Butel and L. S. Richardson, unpublished data); stable lines of GMK cells derived after infection by WT virus and six ts mutants of SV40, designated GMK/WT, GMK/A28, GMK/B401, GMK/B409, GMK/B410, GMK/B415, and GMK/B416. The derivation and characteristics of these cell lines will be described in greater detail in the Results section.

All of the transformed cells were cultured at 37 C (unless otherwise noted) in Eagle medium supplemented with 5% fetal bovine serum (FBS) (Grand Island Biological Co. [GIBCO], Grand Island, N.Y.). Cells were maintained in Eagle medium containing 2% FBS. Medium for culturing TC-7 cells was further supplemented with 10% tryptose phosphate broth, 2% BME vitamins (GIBCO), and 0.25% glucose, whereas the maintenance medium contained 2% FBS and 10% tryptose phosphate broth. Primary GMK cells were grown in Melnick lactalbumin hydrolysate medium supplemented with 2% FBS. Melnick lactalbumin hydrolysate medium containing 10% FBS was used to culture primary human embryonic kidney cells. In addition, all media used contained 100 U of penicillin and 100 μg of streptomycin per ml as well as 0.075% sodium bicarbonate.

Immunofluorescence techniques. Cells to be monitored for the presence of SV40 T or V antigens were grown on 15-mm round cover glasses. At 48 to 72 h postseeding, the cells were harvested, fixed in acetone, and stained as previously described (29). Fluorescein-labeled anti-SV40 monkey serum and acetone fixation for 10 min were used to screen cells for the presence of V antigen.

Saturation density experiments. Saturation density experiments were performed according to the method of Brugge and Butel (3), with the exception that the growth curves were performed in medium containing either 10 or 2% FBS.

Colony formation. (i) In soft agar. Experiments designed to measure the extent of colony formation in soft agar were performed according to the method of Macpherson and Montagnier (19) as previously described (3).

(ii) On plastic. Cells growing at the permissive temperature were tested for their ability to form discrete multilayered foci on plastic surfaces, according to the method of Brugge and Butel (3). The number of deeply staining foci (excluding areas of lightly stained cells) was counted to calculate the number of transformed colonies per plate.

Uptake of hexose. The ability of the various cell lines to transport the hexose molecule, deoxyglucose, was determined as described (3), with the exception that each monolayer of cells was incubated at 37 C in 2 ml of phosphate-buffered saline containing 1 μCi of [^{3}H]deoxyglucose per ml.

Agglutination by ConA. Cells growing at the permissive temperature (37 C) were washed five times with warm 0.2 M phosphate-buffered saline without Ca^{2+} and Mg^{2+}, pH 7.4, and removed from the plastic surface by gentle shaking. The cells were pelleted by low-speed centrifugation (800 rpm, 10 to 15 min) and resuspended in phosphate-buffered saline to a final concentration of 5×10^6 to 10×10^6 cells/ml. A 0.1-ml aliquot of each suspension was mixed with an equal volume of ConA at varying concentrations (10, 100, 250, and 500 μg/ml) in the wells of a flat-bottomed microtiter plate (Micro Test II, Falcon Plastics, Oxnard, Calif.). The reaction was carried out at room temperature for 10 min with continual shaking. The degree of agglutination obtained was evaluated by microscopic examination of the test wells.

Rescue of virus from SV40-transformed cells. Cellular DNA was extracted from the transformed simian cells using a modified Marmur extraction procedure and was passed in TC-7 cells according to the method of Boyd and Butel (2). The titer of virus in the positive harvests was increased by a second passage in TC-7 cells. Likewise, 12 to 20 virus plaques appearing in the initial infectivity assays of the harvested cultures were picked at random and passaged once in TC-7 cells. The titers of all the rescued virus isolates were determined at 33 and 40.5 C.

Extraction of infectious SV40 DNA. Viral DNA was isolated by the Hirt extraction procedure (13) 5 to 7 days postinfection from TC-7 cells that had been infected with WT virus at an MOI of 1 PFU/cell. The viral DNA-containing supernatant was then treated with self-digested Pronase (100 μg/ml) for 30 min at 37 C, extracted two to three times with redistilled phenol at 20 C, and dialyzed at 4 C against 1× SSC-E (0.15 M NaCl, 0.015 M sodium citrate, and 0.001 M EDTA) until all traces of phenol were removed. The amount of infectious DNA present in the extract was determined by plaque assay in TC-7 cells in the presence of an equal volume of 1 mg of DEAE-dextran (Pharmacia, Uppsala, Sweden; molecular weight, 2×10^6) per ml (26).

Superinfection studies. Cells grown at 37 C were trypsinized and seeded into 25-cm^2 plastic flasks (Falcon Plastics, Oxnard, Calif.) and incubated at 37 C. When the cultures reached confluency, each cell line was infected with SV40 WT virus or adenovirus type 7 (Hu) at an MOI of 5 PFU/cell. After an adsorption period of 1.5 h at 37 C, the flasks were washed two times with Tris-buffered saline and incubated for an additional 30 min at 37 C in the presence of rabbit anti-SV40 serum (titer 1:320) (Microbiological Associates, Bethesda, Md.) or rabbit anti-adenovirus type 7 (Hu) serum to neutralize residual virus. The cells were then washed three times with Tris-buffered saline, flooded with maintenance media, and incubated at 37 C. Cultures were also infected with SV40 WT DNA at an MOI of 0.1 PFU/cell in the presence of 1 mg of DEAE-dextran per ml. After a 45-min adsorption period the cells were washed and incubated as described above. Cultures were harvested at 6 and 96 h postinfection and assayed for the presence of infectious SV40 or adenovirus in TC-7 or human embryonic kidney cells, respectively.

RESULTS

Establishment of SV40-transformed monkey cell lines. Confluent cultures of primary GMK cells were incubated with WT SV40 as well as with representative mutants of the A and B complementation groups, at an MOI of 0.001 PFU/cell. Subsequent to a 72-h incubation at room temperature, the infected cells were subcultured at 37 C in the presence of SV40 neutralizing antisera. Cultures, mock infected with Tris-buffered saline, were processed in an identical manner. At approximately 20 to 25 days postinfection (at passage level 5 or 6), cytopathic effect was observed in all of the infected cultures. Cell degeneration progressed rapidly until less than 10 discrete colonies/75-cm^2 culture flask remained intact. There was no observable difference in the number of colonies surviving after infection by WT or any of the ts mutants. The resultant cell lines were established by the subcultivation of these surviving colonies. At passage level 10, SV40 neutralizing antiserum was removed from the culture fluids. The mock-infected cultures could not be maintained beyond eight in vitro passages.

Synthesis of viral antigens and release of infectious virus by established cell lines. The cultures of simian cells derived after infection were monitored routinely for the presence of the virus-specific T and V antigens as well as for the release of infectious virus. A summary of this data is presented in Table 1. The cells became greater than 99% positive for the expression of T antigen after approximately 10 to 20 in vitro passages and continued to synthesize large amounts of T antigen at all subsequent passage levels. The frequency of V antigen production, on the other hand, was low, never involving more than 10% of the cells. The percentage of cells producing V antigen steadily decreased until no antigen-positive cells could be detected (approximately 20 to 30 passages).

When cultures of the monkey cell lines were lysed by freezing and thawing and the supernatant fluids were assayed, levels of infectious virus could be detected. Although the quantity of virus released seemed to decrease gradually, small amounts of virus continued to be recovered even at high passage levels (passages 50 to 60) at which no V antigen-positive cells could be observed. The growth properties of the virus released from these cells resembled those of the original virus inoculum.

Since it would not be possible to determine the growth characteristic of virus rescued from the transformed cultures while they continued to release virus spontaneously, SV40 neutraliz-

TABLE 1. *Antigen synthesis and virus production by SV40-transformed simian cells*[a]

Cell line	Antigen production[b]			Virus release		Phenotype of virus released[b]
	Passage no.	% T positive	% V positive	Passage no.	PFU/cell	
GMK/WT	18	100	2-5	21	0.13	
	24	100	0	25	0.0008	
	34	100	0	31	0.068	WT
	45	100	0	+Antisera[c]		
				40	<0.00001	
				54	<0.00001	
GMK/A28	8	80	5	10	0.014	
	16	100	2-5	25	0.54	
	30	100	1	30	0.014	TS
	42	100	0	+Antisera		
	60	100	0	38	0.009	
	69	100	0	53	0.00026	
GMK/B401	10	100	10	50	0.0064	
	22	100	2-5			ND
	54	100	0			
GMK/B409	11	100	10	25	0.3	
	32	100	1	35	0.22	ND
				54	0.0016	
GMK/B410	10	100	8	14	0.0028	
	20	100	2-5	65	<0.00001	
	44	100	0	74	<0.00001	TS
	89	100	0	108	<0.00001	
	114	100	0	120	<0.00001	
GMK/B415	10	100	0.1	14	0.004	
	20	100	0	25	0.0008	
	28	100	0	+Antisera		TS
	39	100	0	36	<0.00001	
	52	100	0	46	<0.00001	
				53	<0.00001	
GMK/B416	15	100	2.5	38	0.006	
	27	100	0.1	51	0.0004	ND
	35	100	0	59	0.0002	
	52	100	0			

[a] Cultures of primary GMK cells were infected with SV40 WT as well as with mutants of the A and B complementation groups at an MOI = 0.001 PFU/cell. After 72 h at room temperature, the cells were subcultured at 37 C in the presence of SV40 neutralizing antisera. Cultures were mock-infected with Tris-buffered saline and processed in an identical manner.

[b] T, Tumor antigen; V, viral capsid antigen; TS, temperature sensitive; ND, not done.

[c] Cells were passaged eight times in the presence of SV40 neutralizing antisera. Cells were subsequently monitored for the release of infectious virus.

ing antiserum was reintroduced into the culture fluids of several cell lines for five to eight passages. When the cells were retested for spontaneous virus release, no infectious virus was detected in the GMK/WT and GMK/B415 cell lines. Although the amount of virus released by the GMK/A28 cells was reduced, the cells continued to shed infectious virus. These cells are now being passaged a second time in the presence of neutralizing antiserum. Of the seven cell lines initially developed, four were selected for further analysis of their growth characteristics.

Characterization of the cellular growth patterns of SV40-transformed simian cells. (i) Morphology. A culture of uncloned, primary monkey kidney cells contains a morphologically heterogeneous population of cells. During the evolution of the transformed cell lines, certain cell types appeared to be selected, presumably at random, such that each resulting cell line had a characteristic morphological appearance. Attempts to clone these lines have been largely unsuccessful.

The morphologies of the GMK/B410 and GMK/B415 cell lines, grown at the permissive and nonpermissive temperatures, are illustrated in Fig. 1. The GMK/WT and GMK/A28 lines were similar in morphology to the GMK/B415 line and are not shown. After becoming greater than 99% T antigen positive, all of the lines retained the characteristic growth pattern

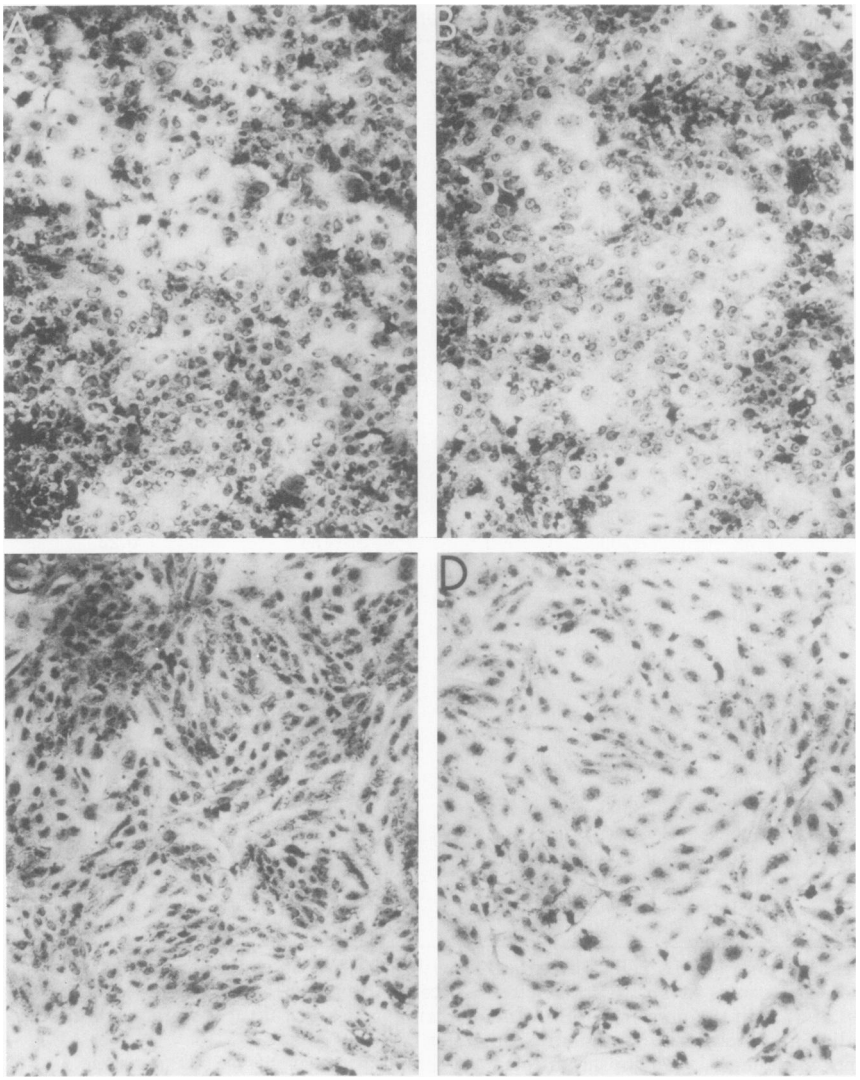

FIG. 1. *Morphological appearance of SV40-transformed simian cells at the permissive (37 C) and nonpermissive (40.5 C) temperatures stained with hematoxylin. (A) GMK/B410 cells at 37 C; (B) GMK/B410 cells at 40.5 C; (C) GMK/B415 cells at 37 C; (D) GMK/B415 cells at 40.5 C.*

of normal, untransformed cells. The cells appeared contact inhibited and, consequently, did not form foci or multiple layers of cells. In addition, repeated passage (three to four times) of the cells at the nonpermissive temperature had no apparent effect on the growth behavior of any of the cell lines tested.

(ii) Saturation density in high and low serum. SV40-transformed hamster, mouse, or human cells, unable to respond to density-dependent growth controls, continue to divide until necessary growth factors have been depleted from the media or until the cells slough from the culture surface (3). As a result of this loss of contact inhibition, the cells grow to higher saturation densities than their normal cellular counterparts. Saturation density experiments in high and low serum concentrations were performed with the SV40-transformed simian cells in an effort to determine if they possessed any altered growth capabilities.

Figures 2A and B illustrate the growth curves of various normal and SV40-transformed monkey cells in the presence of high (10%) or low (2%) serum concentrations, respectively. When the cells were grown in 10% serum, the maximum cell densities attained ranged from 12×10^4 to 24×10^4 cells/cm^2. None

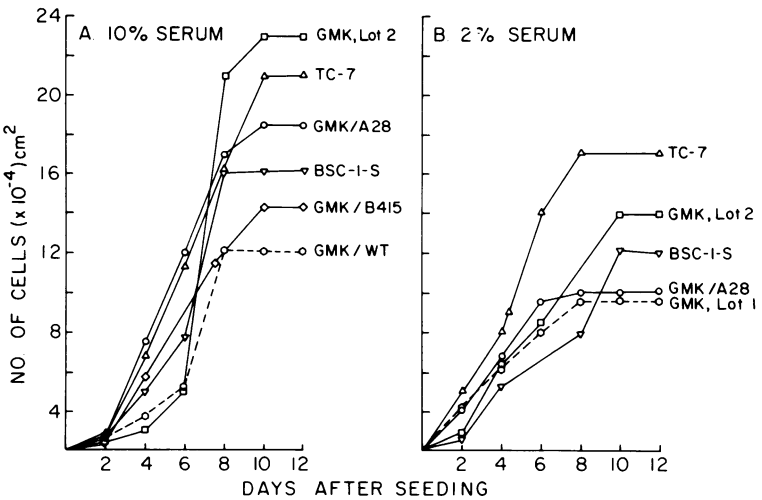

FIG. 2. *Growth curves of normal and SV40-transformed simian cells in high (10%) and low (2%) concentrations of serum at the permissive (37 C) temperature were performed as described in Materials and Methods for saturation density experiments. (A) Normal (GMK-Lot 2, TC-7) and SV40-transformed (GMK/WT, GMK/A28, GMK/B415, BSC-1-S) simian cells in 10% serum; (B) normal (GMK-lot 1, GMK-lot 2, TC-7) and SV40-transformed (GMK/A28, BSC-1-S) simian cells in 2% serum.*

of the SV40-transformed cells grew to higher saturation densities than normal monkey cells. The highest densities observed, in fact, were those of the two normal lines used.

Reducing the serum concentration to 2% failed to give the transformed cells a selective growth advantage. The maximum saturation densities attained by all of the cell lines were somewhat reduced when compared to those seen when 10% FBS was used (Fig. 2B). The basic growth patterns, however, were not significantly altered. Again, two of the normal cell lines reached higher densities than either of the transformed cultures. It will be noted that variances in the saturation densities attained by independently derived lots of primary GMK cells did occur.

In an effort to determine if the growth properties of the SV40-transformed simian cells would be altered in any way by the thermal inactivation of the gene A protein, the WT and mutant cells were grown concomitantly at the permissive (37 C) and nonpermissive (40.5 C) temperatures. The resulting growth curves (Fig. 3) illustrate that the level of saturation density achieved by each cell line tested was unaffected by the temperature of incubation. There was no significant reduction in the saturation densities attained at the nonpermissive temperature. In fact, with the exception of one cell line, GMK/B415, all of the transformed cell lines grew to a higher density at 40.5 than at 37 C. In confirmation of observations made in Fig. 2A and B, all of the transformed lines grew

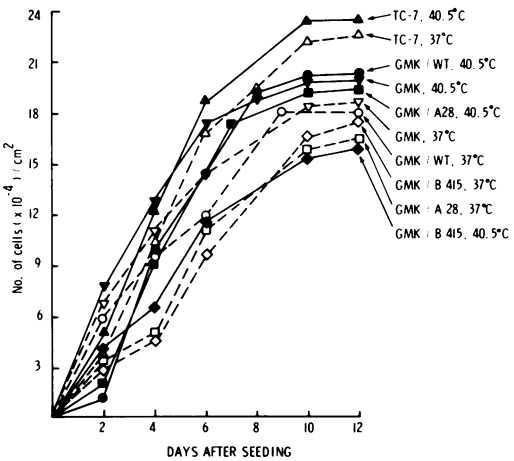

FIG. 3. *Growth curves of normal (GMK, TC-7) and SV40-transformed (GMK/WT, GMK/A28, GMK/B415) simian cells at the permissive (37 C) and nonpermissive (40.5 C) temperatures.*

to densities lower than those attained by the normal TC-7 line at both temperatures.

(iii) Colony formation. SV40-transformed cells from nonpermissive or semipermissive species can form colonies in soft agar as well as produce deeply staining, multilayered foci (or colonies) of cells on plastic surfaces (3). These properties also appear to be under the control of the gene A protein since mouse, hamster, and human cells transformed by the tsA mutants fail to exhibit these transformed properties

when grown under restrictive conditions. Experiments were performed to assess the ability of the mutant- and WT-transformed permissive cells to form colonies on plastic surfaces and in soft agar at both the permissive and nonpermissive temperatures. When monkey cells growing at 37 C were plated onto plastic surfaces and incubated at 37 or 40.5 C they were able to adhere, divide, and form colonies of cells a single layer thick. None of the SV40-transformed cell lines, however, was able to produce colonies that resembled the multilayered, deeply staining foci seen with transformed hamster, mouse, or human cells; the colonies of simian cells contained cells that were completely contact inhibited and therefore stained only lightly. All of the cell lines plated with equal efficiencies at 37 and 40.5 C, indicating that none of the cell lines was temperature sensitive for normal growth (Table 2).

TABLE 2. *Plating efficiencies of normal and SV40-transformed simian cells on plastic surfaces*[a]

Cell line	Plating efficiency (%)		Ratio (P/NP)
	Permissive temp (P)	Nonpermissive temp (NP)	
TC-7	86.0	80.7	1.1
GMK/WT	61.6	55.1	1.1
GMK/A28	75.9	62.3	1.2
GMK/B410	63.2	50.0	1.3
GMK/B415	68.6	55.3	1.2

[a] Cells growing at the permissive temperature (P) were trypsinized and seeded into 35-mm plastic petri dishes at a concentration of 10^2 to 10^3 cells/plate in 2 ml of Eagle medium containing 10% FBS. Three days after seeding, the cells were stained as described in Materials and Methods and the number of attached cells was determined.

When comparable experiments were performed in soft agar, neither the normal nor the SV40-transformed cells were able to form discernible colonies at either 37 or 40.5 C. Although there was no known phenotypically transformed monkey cell line to include in the experiment, BALB/WT-VLM cells (a line of mouse cells transformed by WT SV40 virus) did form distinct colonies under the experimental conditions.

Expression of SV40-induced T antigen. As noted previously, the SV40-transformed monkey cells became greater than 99% positive for the expression of T antigen after 10 to 20 in vitro passages. In subsequent passages (see Table 1), all of the cells in each of the cultures continued to synthesize T antigen at a uniform intensity of fluorescence. Figure 4 illustrates the typical patterns of fluorescence seen with both the WT- and mutant-transformed cells at 37 C. To determine whether there was any temperature-induced modulation of T antigen production, the cells were passaged three times at 40.5 C and re-examined by immunofluorescence. None of the cell lines, including the GMK/A28 line, showed any alteration in the production of an immunologically identifiable T antigen after three passages at the nonpermissive temperature.

Rescue of infectious virus from SV40-transformed simian cells. Virus rescue by the DNA transfection technique was attempted to determine if those cell lines that were no longer spontaneously releasing infectious virus contained an intact, infectious viral genome whose properties resembled those of the inoculum virus. The results of those experiments are summarized in Table 3. Of the three lines tested, virus was successfully rescued from the GMK/

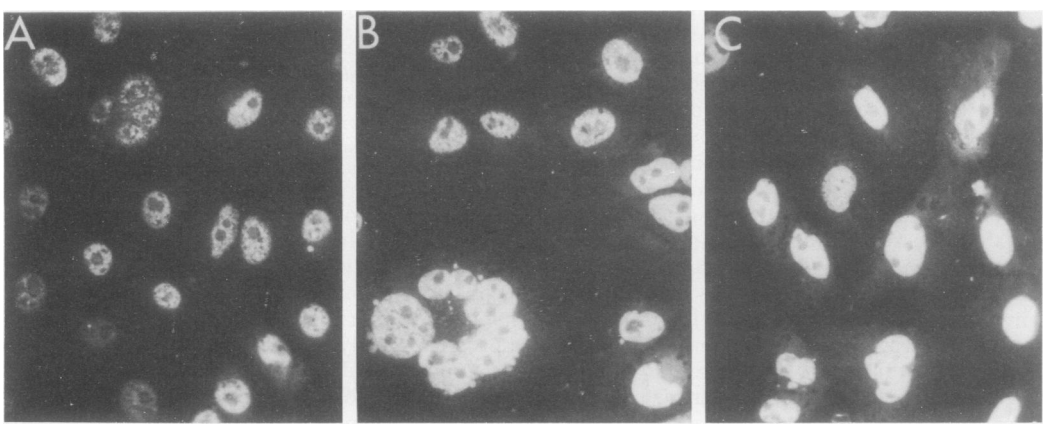

FIG. 4. *Immunofluorescence photomicrographs of SV40 T antigen in the nucleus of SV40-transformed simian cells. (A) GMK/WT cells at 37 C; (B) GMK/A28 cells at 37 C; (C) GMK/B415 cells at 37 C. ×240.*

TABLE 3. *Rescue of virus from SV40-transformed simian cells*[a]

Source of cellular DNA	No. of positive cultures/no. of inoculated cultures		Efficiency of plating[b]	
	DNA + DEAE	DNA − DEAE	Total population of rescued virus	Plaque-purified isolates
GMK/WT	0/20	0/20		
GMK/B410	10/10	0/10	$<1.0 \times 10^{-4}$	$<1.2 \times 10^{-4}$
GMK/B415	6/20	0/20	$<2.7 \times 10^{-5}$	$<4.3 \times 10^{-5}$

[a] Virus was rescued from the transformed simian cells by the DNA transfection technique as described in Materials and Methods. The rescued virus, which had been passed two times through TC-7 cells, was titered by the standard plaque assay technique at the permissive (33 C) and nonpermissive (40.5 C) temperatures.

[b] Expressed as (PFU per milliliter at 40.5 C)/(PFU per milliliter at 33 C).

B410 and GMK/B415 lines. The ratio of the efficiency of plating at the nonpermissive and permissive temperatures of the virus isolated from either line was $<10^{-4}$, indicating that the rescued virus was indeed temperature sensitive.

No virus has been rescued from the GMK/WT cells, but additional recovery attempts are in progress. Virus rescue from the GMK/A28 cells was not performed since these cells have continued to produce small amounts of infectious virus.

Cell surface alterations. (i) Agglutination by ConA. Certain plant lectins, such as ConA and wheat germ agglutinin, have been found to preferentially agglutinate cells transformed by a variety of DNA and RNA tumor viruses (23). This agglutinability has been correlated with the loss of specific growth controls, and existing evidence strongly suggests that the continual expression of one or more viral genes may be necessary to maintain the surface alteration(s) responsible for the agglutination reaction (23). Brugge and Butel (unpublished data) have found that mouse cells transformed by the tsA30 mutant of SV40 (Balb/A30 cells) were agglutinated by ConA when grown at the permissive temperature but failed to agglutinate when passaged at the nonpermissive temperature prior to testing. In contrast, mouse cells transformed by WT virus were agglutinated at both temperatures.

The SV40-transformed simian cells were tested for ConA agglutinability to determine whether those cells possessed surface alterations comparable to those found in SV40-transformed mouse cells (Table 4). As noted previously, Balb/A30 cells grown at 33 C were completely agglutinated at a concentration of 500 μg of ConA per ml. Cells grown at 39.5 C, however, failed to agglutinate when similar concentrations of the lectin were used, mirroring the behavior of normal BALB-3T3 cells. Normal TC-7 cells were not agglutinated by any of the concentrations of ConA used. Similarly, none of the SV40-transformed cells were agglutinated when exposed to even the highest levels of ConA. The failure of these cells to be agglutinated demonstrates the absence of yet another marker associated with classical viral transformation.

(ii) Uptake of hexose. An additional property of transformed cells, which lends support to the theory that transformation of cells by DNA or RNA tumor viruses results in characteristic surface membrane alterations, is the enhanced ability of such cells to transport specific hexose molecules across the plasma membrane (41). BALB-3T3 cells transformed by WT SV40 show a 2.5- to 3.5-fold greater uptake of the hexose deoxyglucose than normal BALB-3T3 cells (15). In addition, both mouse and hamster cells that have been transformed by a tsA mutant of SV40 exhibit a temperature-dependent hexose transport (3).

TABLE 4. *Agglutination by ConA of SV40-transformed mouse and simian cells*[a]

Cell line	Temp (C)	Degree of agglutination[b]			
		10 μg/ml[c]	100 μg/ml	250 μg/ml	500 μg/ml
BALB-3T3	33	0	0	0	0
Balb/A30[d]	33	+	++	+++	++++
	39	0	0	0	0
TC-7	37	0	0	0	0
GMK/WT	37	0	0	0	0
GMK/A28	37	0	0	0	0
GMK/B410	37	0	0	0	0
GMK/B415	37	0	0	0	0

[a] Cells growing at the permissive temperature were washed five times with warm Ca^{2+}- and Mg^{2+}-free 0.2 M phosphate-buffered saline (pH 7.4). The cells were removed from the plastic surface by gentle shaking. The cells were pelleted and resuspended to a final concentration of 5×10^6 to 10×10^6 cells/ml. A 0.1-ml aliquot of ConA at varying concentrations (10, 100, 250, and 500 μg/ml) was mixed with an equal volume of the cell suspension in the wells of a flat-bottomed microtiter plate. After 10 min of agitation, the amount of agglutination was scored by microscopic examination of the wells.

[b] 0, No agglutination; ++++, 100% agglutination.

[c] Concentration of ConA.

[d] A line of BALB-3T3 cells transformed by the tsA30 mutant, which is temperature sensitive for the maintenance of the transformed phenotype (Brugge and Butel, unpublished data).

Cultures of normal and WT- or mutant-transformed simian cells grown at 37 or 40.5 C were monitored for the ability to transport 2-deoxy-D-glucose in a further attempt to detect the presence of any surface membrane alterations. The two transformed lines tested showed a slightly increased hexose transport over that of the normal cells (Table 5). The differences, however, are not great enough to warrant the conclusion that those cells that contain viral information have an altered sugar transport system. In addition, there was no apparent effect of temperature on the amount of hexose taken up by any of the cultures, including the GMK/A28 line (permissive/nonpermissive temperature = 0.7 to 1.0). In summary, there appears to be no measurable effect of the viral genome on the synthesis or arrangement of surface membrane components, which results in an alteration in the hexose transport system.

Susceptibility of normal and SV40-transformed simian cells to superinfection with virus or infectious nucleic acid. The various simian lines were monitored for their ability to support the replication of superinfecting homologous and heterologous viral genomes. The majority of SV40-transformed simian cell lines tested in previous studies were found to be resistant to superinfection by mature virus (5, 20, 30, 34, 35), but remained susceptible to SV40 infectious DNA (17, 34, 35). One cell line, however, was refractory to both intact virus and viral DNA (5).

Human adenoviruses undergo an abortive infection in most established simian cell lines.

TABLE 5. *Uptake of deoxyglucose by normal and SV40-transformed monkey cells*[a]

Cell line	Uptake of deoxyglucose (counts/min per 10^5 cells)		Ratio (P/NP)
	Permissive temp (P) (37 C)	Nonpermissive temp (NP) (40.5 C)	
GMK	1,765	2,418	0.7
TC-7	1,089	1,363	0.8
GMK/WT	2,304	2,594	0.9
GMK/A28	2,777	2,747	1.0

[a] Seventy-five percent confluent monolayers of cells in 35-mm petri dishes were washed two to three times with phosphate-buffered saline (PBS) (pH 7.2) warmed to 37 C. A 2-ml solution of PBS containing 1 μCi of 2-[^{3}H]deoxyglucose per ml was added to each plate. The cells were incubated in a 37 C water bath for 10 min. Triplicate samples of the cells were then washed with cold PBS and scraped into 1 ml of PBS, and a sample of 0.1 ml was assayed for radioactivity. The results are expressed as an average of the three cultures. Cell counts were determined on duplicate cultures maintained under identical conditions.

The early events in the adenovirus replicative cycle, as well as the synthesis and polyadenylation of late mRNA, occurs normally, whereas viral capsid proteins are present in greatly reduced quantities (1, 9, 11, 12, 28, 31). Most recently, the defective step in the infectious cycle has been shown to involve an inability of the late mRNA to form polyribosomic complexes (12, 22). This block in adenovirus replication can be overcome by co-infection with SV40 (27). A productive adenovirus cycle will also occur in most SV40-transformed simian cells (5, 34). Recently, it has been demonstrated that high-salt extracts of ribosomes from SV40-transformed monkey cells contain a factor that can catalyze the binding of late adenovirus mRNA to ribosomes with the result that 80S initiation complexes are formed (22).

In this study, both SV40 and adenovirus type 7 (Hu), as well as SV40 DNA, were used to characterize the newly derived simian cells with respect to their susceptibility to superinfection. GMK/PARA-7-1 and TC-7 cells were included as control lines. The results of these superinfection studies are summarized in Table 6. Whereas the normal simian line, TC-7, replicated both SV40 virus and DNA to high titers, it failed to support the growth of the human adenovirus. The GMK/PARA-7-1 line proved highly susceptible to adenovirus as well as to SV40 virus and viral DNA. In contrast, the SV40-transformed simian lines were completely resistant to superinfection by mature SV40 virus. Likewise, when infectious SV40 DNA was used all of the lines remained refractory, indicating that the block in replication was not solely at the level of adsorption, penetration, or uncoating.

Adenovirus replicated efficiently in three of the four transformed cell lines, GMK/WT, GMK/A28, and GMK/B415, titers of 10^5 to 10^6 PFU/ml being obtained at 96 h postinfection. The GMK/B410 line, however, was unable to support the complete replication of adenovirus 7. Immunofluorescence tests were performed to determine whether adenovirus 7 T and/or V antigens were being synthesized in this cell line. Approximately 10% of the cells in each of the cultures, including the GMK/B410 cells, were positive for both adenovirus T and V antigens. Additional experiments are being performed to better characterize the block in adenovirus replication in the GMK/B410 cells.

The transformed simian cells were further examined to determine if there was any effect of temperature on the ability of the cells to complement the growth of adenovirus. The cells were passaged two times at 33 or 40.5 C before the experiment was initiated. The super-

infected cells were incubated at 33 or 40.5 C for 96 or 72 h postinfection, respectively. The titers obtained are summarized in Table 7. As noted in the previous experiments, effective complementation was obtained with the GMK/WT, GMK/A28, GMK/B415, and GMK/PARA-7-1 cell lines. In addition, the SV40 helper activity remained functional regardless of the temperature of incubation. No significant differences in titer were obtained when the experiment was performed at the nonpermissive temperature. A negligible increase in the adenovirus titer was obtained at both temperatures after superinfection of the GMK/B410 cell line.

DISCUSSION

In general, the SV40-transformed simian cells derived for this study can be distinguished from the parental GMK line by several characteristics, including an enhanced life span, the constitutive production of SV40 T antigen, a resistance to superinfection by SV40 virus or viral DNA, and, with one exception, an ability to complement the growth of human adenovirus type 7. Similar patterns were observed whether the cell lines were derived after infection by a ts mutant or a WT virus. Each of the properties described was found to be expressed independent of the temperature of incubation, indicating that the gene A protein does not appear to be essential for the continued expression of these properties. Unlike the SV40-transformed mouse, hamster, and human cells, which possess many of the classical properties commonly associated with transformation, the SV40-transformed simian cells failed to exhibit any consistent or dramatic alteration in patterns of growth control or membrane function relative to primary or stable lines of normal monkey cells.

As mentioned previously, the isolation of

TABLE 6. *Ability of normal and SV40-transformed simian cells to support the replication of SV40 virus, SV40 DNA, or adenovirus 7*[a]

Cell line	Virus yields (PFU/10^6 cells) p.i. with:					
	SV40 WT virus[b]		SV40 WT DNA[b]		Adenovirus 7[c]	
	6 h p.i.	96 h p.i.	6 h p.i.	96 h p.i.	6 h p.i.	96 h p.i.
TC-7	$<1.0 \times 10^1$	1.0×10^8	2.7×10^3	6.0×10^7	5.0×10^1	$<1.0 \times 10^1$
GMK/PARA-7-1[d]	$<1.0 \times 10^1$	3.0×10^7	$<1.0 \times 10^1$	6.0×10^6	$<1.0 \times 10^1$	1.4×10^6
GMK/WT	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	1.5×10^2	4.0×10^6
GMK/A28	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	8.0×10^2	1.5×10^6
GMK/B410	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	1.4×10^3	2.5×10^2
GMK/B415	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	1.0×10^7

[a] Cultures of each of the cell lines were exposed to 5 PFU of SV40 or adenovirus type 7 per cell or 0.1 PFU of SV40 DNA per cell. Replicate cultures were harvested by freezing and thawing at 6 and 96 h postinfection (p.i.). The amount of virus per culture was determined by plaque assay techniques as described in Materials and Methods.

[b] Assayed in TC-7 cells.

[c] Assayed in human embryonic kidney cells.

[d] Primary GMK cells transformed in vitro by PARA-adenovirus 7.

TABLE 7. *Effect of temperature on the enhancement of adenovirus replication by normal and SV40-transformed simian cells*[a]

Cell line	Adenovirus 7 yields (PFU/10^6 cells)				Ratio of virus yields (P/NP)
	Permissive temp (P)		Nonpermissive temp (NP)		
	6 h p.i.	96 h p.i.	6 h p.i.	96 h p.i.	
TC-7	2.5×10^2	$<1.0 \times 10^1$	1.5×10^2	$<1.0 \times 10^1$	
GMK/PARA-7-1	$<1.0 \times 10^1$	4.5×10^6	5.0×10^1	2.8×10^6	1.6
GMK/WT	1.5×10^3	2.4×10^7	4.2×10^2	9.0×10^6	2.7
GMK/A28	1.4×10^3	3.3×10^7	9.0×10^2	9.5×10^6	3.5
GMK/410	1.3×10^4	4.5×10^4	1.0×10^4	7.0×10^4	
GMK/B415	8.5×10^1	3.0×10^7	1.9×10^2	1.2×10^7	2.5

[a] Replicate cultures of cells were infected with 5 PFU of adenovirus type 7 per cell and incubated at the permissive or nonpermissive temperature. Cultures were harvested by freezing and thawing at 6 and 96 h postinfection (p.i.). The amount of infectious virus present was determined by plaque assay in human embryonic kidney cells.

SV40-transformed simian cells is, at best, a rare event which is difficult to quantitate. Consequently, little is known about the mechanism of conversion of primary cells to the transformed state. Important facts such as the type of cell that becomes transformed, the properties of the virus that initiates the conversion, and the mechanism controlling the expression of the SV40 genome in the absence of a classical transformed phenotype remain undetermined. However, the biological properties of a variety of transformed cells derived in several different laboratories suggest that the nature of the infecting virus, as well as the host cell, plays an important role in determining the outcome of the interaction.

In general, the cell lines isolated to date can be divided into three basic classes. The first class is comprised of a cell line (T-22) that possesses the characteristics of a culture transformed by a defective viral genome; that is, the cells can support the replication of a superinfecting SV40 genome but fail to yield infectious virions by the fusion (14, 34) or DNA transfection techniques (2). In fact, these cells were derived after exposure to a heavily irradiated virus population. Cells (GMK/EVa) that have the ability to replicate superinfecting SV40 genomes as well as produce infectious progeny after fusion with normal GMK cells represent a second class of cells that have been isolated (18, 35). This type of cell line could have been derived from a rare permissive cell that has irreversibly integrated and partially repressed an intact, nondefective viral genome. Finally, some cell lines (BSC-1-S, P-58-2) have been derived that are resistant to superinfection by SV40 virus or viral DNA, yet yield infectious virus by various rescue techniques (20, 34). The experimental evidence available indicates that all of the cultures isolated in this study appear to fall into the latter category. The most plausible explanation is that, first, each line arose from a variant cell in the heterogeneous GMK population, which is either mutated or in a different stage of development such that it is deficient in some factor essential for the replication of the viral genome, and, second, a nondefective viral genome has become stably associated with the host cell DNA.

Recent experiments using semi- and nonpermissive cells transformed by ts mutants of SV40 have suggested that the continuous expression of the A gene is required to maintain the transformed phenotype (3, 17, 21, 25, 39). This finding represents the first direct evidence that the mere integration of the viral genome into the host cell is not sufficient to cause transformation; rather, some active participation of a viral gene product is required for this event. Cells of mouse, hamster, rat, and human origin behaved similarly, indicating that the gene A protein can interact in a similar manner in cells that are either semipermissive or nonpermissive for SV40 replication. In the lytic infection, the gene A protein, which may in fact be T antigen (24, 37, 38), has been shown to be directly required for the initiation of viral DNA replication (36). Preliminary evidence has also indicated that cellular DNA synthesis is not enhanced after infection of normal cells by the tsA mutants at 42.5 C (6).

Based on this evidence, a working model has been devised to explain the mechanism of transformation of semi- and nonpermissive cells by SV40 (Fig. 5). Briefly, this model predicts that the SV40 gene A product can also function either directly or indirectly to initiate cellular DNA replication and that an alteration in the specific sequence of replication can lead to aberrant patterns of growth and result in expression of the transformed phenotype. In this model there are two factors that contribute to the development of the transformed state. The first, the site of integration of the viral genome in the cellular chromosome, would determine if or when within the cell cycle the viral genome is expressed and the gene A protein (V_I) is produced. The second, the target site of this protein, would determine the degree to which the normal cell regulatory functions are perturbed. The activity of the gene A protein might be restricted to the initiation site of the integrated viral genome (V_0) or the regions in the cellular genome that are homologous to it. In addition, or alternatively, the V_I might be able to interact with the normal cellular initiation site(s) ($C_{0-1,2}$), during which time the cell initiator (C_I) may possibly be rendered nonfunctional.

Nothing is known about the state of the viral genome in the transformed simian cells. Since T antigen appears to be produced constitu-

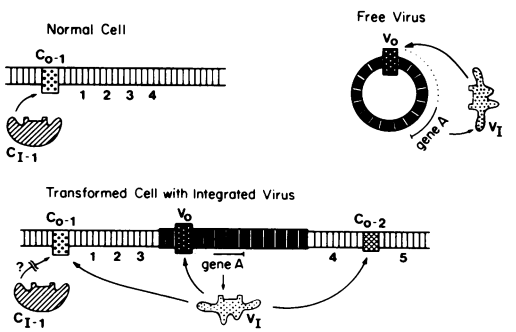

FIG. 5. *Model for the mechanism of transformation by SV40. See Discussion for specific details.*

tively in greater than 99% of the cells, a relatively stable association between viral and cellular genetic material probably exists. However, the viral DNA could be linearly integrated into cell DNA by covalent or alkalilabile linkages or it could be present in a nonlinear state in close association with the cellular chromosome.

The results of this study suggest that, although the cells were stably altered in several parameters, many of the phenotypic changes that occur in SV40-transformed mouse, hamster, and human cells are not present in these transformed simian cells. However, this does not appear to be the only situation that can exist. At least one line of transformed monkey cells (GMK-EVa) has been shown to have some altered growth properties relative to those of normal cells (7). These cells, which exhibit an increased saturation density and a decreased serum requirement, are also able to grow on monolayers of certain cells as well as form colonies in soft agar.

It becomes apparent, then, that several transformed simian cell lines from each of the classes described must be screened for characteristic phenotypic alterations before any conclusions can be drawn regarding the growth behavior of transformed simian cells in general. It is possible that a spectrum of phenotypic changes can exist and that transformation of simian cells cannot be described as an "all or none" phenomenon. This type of spectrum has, in fact, been demonstrated recently in SV40-transformed rat and mouse cells (32). If a similar situation can be found in transformed monkey cells, then the basic tenets of the model described earlier would also apply in general to the transformed simian cells.

Specifically, however, working models can be devised to explain the observed phenotypic characteristics of the cells derived in the study. The first model, which assumes that a functional gene A product is produced in the transformed simian cells, predicts that the V_I acts on the V_O, which is integrated at or near a normal C_O site, and that, once initiated, the synthesis of DNA continues through the viral genome into the region of cell DNA, triggering a complete round of replication. Since there is no alteration in the sequence of replication of cell DNA, synthesis having been initiated near a normal cell initiator site, the existing regulatory controls remain operative and the cells appear phenotypically normal. However, since cell DNA synthesis is repeatedly stimulated by the interactions of the V_I with the V_O, the natural senescence of the cells is overcome and the cells become "immortal."

If the maintenance of this immortalized state is dependent upon the continued functional integrity of the V_I protein, then theoretically, the GMK/A28 cells should become senescent after continuous passage at the nonpermissive temperature. As indicated previously, each of the cell lines has been passaged up to four times at 40.5 C with no apparent effect on the life span of the cells. However, in the lytic cycle, the A mutants exhibit some functional "leak" at the nonpermissive temperature (40.5 C). To reduce the leak of the gene A protein, Chou and Martin (6) assayed the ability of the A mutants to stimulate host cell DNA synthesis in permissive cells at 42.5 C. It was determined that the tsA mutants were deficient in the enhancement function at the elevated temperature. The results of their experiment suggest that all leak must be abolished to establish the true nature of a mutated protein. Passage of the GMK/A28 cells at a higher temperature might serve to eliminate all activity of the V_I, returning the cells to normal growth patterns and eventual senescence.

A second or alternative model suggests that "immortalization" is an event that occurs independent of the action of the viral genome. Since stable lines of monkey cells can be derived from primary cultures, certain cells in the original heterogeneous population probably possess an innate ability to overcome senescence. An enhanced life span might result from some type of cellular maturation or differentiation which arises from an alteration in the existing growth controls.

It is known that the viral gene A product, which initiates viral DNA replication in the lytic cycle, is required to maintain the transformed phenotype of a number of different cell types. Several lines of evidence have suggested that T antigen may in fact be the gene A protein. This study has shown that the mere presence of an immunologically reactive T antigen in the nucleus of transformed simian cells is not sufficient to bring about classical transformation. The process of cellular transformation of simian cells by SV40 would appear to require other specific interactions between viral and cellular genes and/or gene products that have not as yet been identified.

ACKNOWLEDGMENTS

We acknowledge the interest and support of J. L. Melnick.

This study was supported by Public Health Service research grants CA 10,893 and CA 15,149 from the National Cancer Institute. J. S. Butel is the recipient of Faculty Research Award PRA-95 from the American Cancer Society.

LITERATURE CITED

1. **Baum, S. G., M. S. Horwitz, and J. V. Maizel, Jr.** 1972. Studies of the mechanism of enhancement of human adenovirus infection in monkey cells by simian virus 40. J. Virol. 10:211-219.
2. **Boyd, V. A. L., and J. S. Butel.** 1972. Demonstration of infectious deoxyribonucleic acid in transformed cells. I. Recovery of simian virus 40 from yielder and nonyielder transformed cells. J. Virol. 10:399-409.
3. **Brugge, J. S., and J. S. Butel.** 1975. Role of simian virus 40 gene A function in maintenance of transformation. J. Virol. 15:619-635.
4. **Butel, J. S., and F. Rapp.** 1966. Replication in simian cells of defective viruses in an SV40-adenovirus "hybrid" population. J. Bacteriol. 91:278-284.
5. **Butel, J. S., L. S. Richardson, and J. L. Melnick.** 1971. Variation in properties of SV40-transformed simian cell lines detected by superinfection with SV40 and human adenoviruses. Virology 46:844-855.
6. **Chou, J. Y., and R. G. Martin.** 1975. DNA infectivity and the induction of host DNA synthesis with temperature-sensitive mutants of simian virus 40. J. Virol. 15:145-150.
7. **Eagle, H., G. E. Foley, H. Koprowski, H. Lazarus, E. M. Levine, and R. A. Adams.** 1970. Growth characteristics of virus-transformed cells. Maximum population density, inhibition by normal cells, serum requirement, growth in soft agar, and xenogenic transplantability. J. Exp. Med. 131:863-879.
8. **Enders, J. F.** 1965. Cell transformation by viruses as illustrated by the response of human and hamster renal cells to simian virus 40, p. 113-154. *In* The Harvey lecture series 59. Academic Press Inc., New York.
9. **Feldman, L. A., J. S. Butel, and F. Rapp.** 1966. Interaction of a simian papovavirus and adenovirus. I. Induction of adenovirus tumor antigen during abortive infection of simian cells. J. Bacteriol. 91:813-818.
10. **Fernandes, M. V., and P. S. Moorhead.** 1965. Transformation of African green monkey kidney cultures infected with simian vacuolating virus (SV40). Tex. Rep. Biol. Med. 23:242-258.
11. **Fox, R. I., and S. G. Baum.** 1972. Synthesis of viral ribonucleic acid during restricted adenovirus replication. J. Virol. 10:220-227.
12. **Fox, R. I., and S. G. Baum.** 1974. Posttranscriptional block to adenovirus replication in nonpermissive monkey cells. Virology 60:45-53.
13. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. 26:365-369.
14. **Huebner, K., D. Santoli, C. M. Croce, and H. Koprowski.** 1975. Characterization of defective SV40 isolated from SV40-transformed cells. Virology 63:512-522.
15. **Isselbacher, K. J.** 1972. Increased uptake of amino acids and 2-deoxy-D-glucose by virus-transformed cells in cultures. Proc. Natl. Acad. Sci. U.S.A. 69:585-589.
16. **Kimura, G., and A. Itagaki.** 1975. Initiation and maintenance of cell transformation by simian virus 40: a viral genetic property. Proc. Natl. Acad. Sci. U.S.A. 72:673-677.
17. **Kit, S., T. Kurimura, M. Brown, and D. R. Dubbs.** 1970. Identification of the simian virus 40 which replicates when simian virus 40-transformed human cells are fused with simian virus 40-transformed mouse cells or superinfected with simian virus 40 deoxyribonucleic acid. J. Virol. 6:69-77.
18. **Koprowski, H., F. C. Jensen, and Z. Steplewski.** 1967. Activation of production of infectious tumor virus SV40 in heterokaryon cultures. Proc. Natl. Acad. Sci. U.S.A. 58:127-133.
19. **Macpherson, I., and L. Montagnier.** 1964. Agar suspension culture for the selective assay of cells transformed by polyoma virus. Virology 23:291-294.
20. **Margalith, M., R. Volk-Fuchs, and N. Goldblum.** 1969. Transformation of BSC 1 cells following chronic infection with SV40. J. Gen. Virol. 5:321-327.
21. **Martin, R. G., and J. Y. Chou.** 1975. Simian virus 40 functions required for the establishment and maintenance of malignant transformation. J. Virol. 15:599-612.
22. **Nakajima, K., H. Ishitsuka, and K. Oda.** 1974. An SV40-induced initiation factor for protein synthesis concerned with the regulation of permissiveness. Nature (London) 252:649-653.
23. **Nicholson, Y. L.** 1974. The interactions of lectins with animal cell surfaces, p. 89-190. *In* G. H. Bourne and J. F. Danielli (ed.), International review of cytology, vol. 39. Academic Press Inc., New York.
24. **Osborn, M., and K. Weber.** 1974. SV40: T antigen, the A function, and transformation. Cold Spring Harbor Symp. Quant. Biol. 39:267-276.
25. **Osborn, M., and K. Weber.** 1975. Simian virus 40 gene A function and maintenance of transformation. J. Virol. 15:636-644.
26. **Pagano, J. S., J. H. McCutchan, and A. Vaheri.** 1967. Factors influencing the enhancement of the infectivity of poliovirus ribonucleic acid by diethylaminoethyl-dextran. J. Virol. 1:891-897.
27. **Rabson, A. S., G. T. O'Conor, I. K. Berezesky, and F. J. Paul.** 1964. Enhancement of adenovirus growth in African green monkey kidney cell cultures by SV40. Proc. Soc. Exp. Biol. Med. 116:187-190.
28. **Rapp, F., L. A. Feldman, and M. Mandel.** 1966. Synthesis of virus deoxyribonucleic acid during abortive infection of simian cells by human adenoviruses. J. Bacteriol. 92:931-936.
29. **Rapp, F., S. Pauluzzi, and J. S. Butel.** 1969. Variation in properties of plaque progeny of PARA (defective simian papovavirus 40)-adenovirus 7. J. Virol. 4:626-631.
30. **Rapp, F., and S. C. Trulock.** 1970. Susceptibility to superinfection of simian cells transformed by SV40. Virology 40:961-970.
31. **Reich, P. R., S. G. Baum, J. A. Rose, W. P. Rowe, and S. M. Weissman.** 1966. Nucleic acid homology studies of adenovirus type 7-SV40 interactions. Proc. Natl. Acad. Sci. U.S.A. 55:337-341.
32. **Risser, R., and R. Pollack.** 1974. A nonselective analysis of SV40 transformation of mouse 3T3 cells. Virology 59:477-489.
33. **Robb, J. A.** 1973. Microculture procedures for SV40, p. 517-524. *In* P. F. Kruse and M. K. Patterson (ed.), Tissue culture: methods and applications. Academic Press Inc., New York.
34. **Shiroki, K., and H. Shimojo.** 1971. Transformation of green monkey kidney cells by SV40 genome: the establishment of transformed cell lines and the replication of human adenoviruses and SV40 in transformed cells. Virology 45:163-171.
35. **Swetly, P., G. Barbanti-Brodano, B. Knowles, and H. Koprowski.** 1969. Response of simian virus 40-transformed cell lines and cell hybrids to superinfection with simian virus 40 and its deoxyribonucleic acid. J. Virol. 4:348-355.
36. **Tegtmeyer, P.** 1972. Simian virus 40 deoxyribonucleic acid synthesis: the viral replicon. J. Virol. 10:591-598.
37. **Tegtmeyer, P.** 1974. Altered patterns of protein synthesis in infection by SV40 mutants. Cold Spring Harbor Symp. Quant. Biol. 39:9-15.
38. **Tegtmeyer, P.** 1974. SV40 gene function, p. 55-74. *In* A. S. Kaplan (ed.), Viral transformation and endoge-

nous viruses. Academic Press Inc., New York.
39. **Tegtmeyer, P.** 1975. Function of simian virus 40 gene A in transforming infection. J. Virol. 15:613–618.
40. **Todaro, G., and H. Green.** 1963. Quantitative studies on the growth of mouse embryo cells in culture and their development into established cell lines. J. Cell Biol. 17:299–313.
41. **Tooze, J. (ed.).** 1973. Molecular biology of tumor viruses. Cold Spring Harbor Laboratory. Cold Spring Harbor. N. Y.
42. **Wallace, R.** 1967. Viral transformation of monkey kidney cell cultures. Nature (London) 213:768–770.