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Role of Simian Virus 40 Gene *A* Function in Maintenance of Transformation

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Mouse, hamster, and human cells were transformed at the permissive temperature by mutants from simian virus 40 (SV40) complementation group *A* in order to ascertain the role of the gene *A* function in transformation. The following parameters of transformation were monitored with the transformed cells under permissive and nonpermissive conditions: morphology; saturation density; colony formation on plastic, on cell monolayers, and in soft agar; uptake of hexose; and the expression of SV40 tumor (T) and surface (S) antigens. Cells transformed by the temperature-sensitive (ts) mutants exhibited the phenotype of transformed cells at the nonrestrictive temperature for all of the parameters studied. However, when grown at the restrictive temperature, they were phenotypically similar to normal, untransformed cells. Growth curves showed that the ts *A* mutant-transformed cells exhibited the growth characteristics of wild-type virus-transformed cells at the permissive temperature and resembled normal cells when placed under restrictive conditions. There were 3- to 51-fold reductions in the levels of saturation density, colony formation, and uptake of hexose when the mutant-transformed cells were grown at the elevated temperature as compared to when they were grown at the permissive temperature. Mutant-transformed cells from the nonpermissive temperature were able to produce transformed foci when shifted down to permissive conditions, indicating that the phenotypically reverted cells were still viable and that the reversion was a reversible event. SV40 T antigen was present in the cells at both temperatures, but S antigen was not detected in cells maintained at the nonpermissive temperature. All of the wild-type virus-transformed cells exhibited a transformed phenotype when grown under either restrictive or nonrestrictive conditions. These results indicate that the SV40 group *A* mutant-transformed cells are temperature sensitive for the maintenance of growth properties characteristic of transformation. Virus rescued from the mutant-transformed cells by the transfection method was ts, suggesting that the SV40 gene *A* function, rather than a cellular one, is responsible for the ts behavior of the cells.

The mechanism by which oncogenic viruses mediate cellular transformation is still obscure. Furthermore, it remains to be definitively established that the DNA tumor viruses control events in transformation by the synthesis of one or more specific viral gene products. One approach to dissecting this complex phenomenon involves the use of temperature-sensitive (ts) mutants of a virus. If cells which have been transformed by a ts mutant display a temperature-dependent transformed phenotype, the mutant gene function would be implicated as being involved in the transformation process. "Temperature-dependent transformed phenotype" is defined as a conditional state in which characteristics of transformation are expressed by the transformed cells at a permissive temperature, but are not manifest at a nonpermissive temperature, thereby causing the cells to revert to normal growth behavior.

Since only early viral functions appear to be expressed in simian virus 40 (SV40)-transformed cells (2, 5, 27), it is most reasonable to examine mutants of this virus which are defective in an early gene for their ability to maintain the transformed state. Only one group of early SV40 mutants has been isolated (complementation group *A*). During infection of susceptible monkey cells at the restrictive temperature, group *A* mutants synthesize SV40 tumor (T) antigen and stimulate host cell DNA replication, but fail to produce viral DNA or capsid

antigens (31). More specifically, the gene *A* protein has been found to be required for the initiation of viral DNA synthesis (29).

To determine whether the SV40 gene *A* function is involved in the maintenance of transformation, we transformed normal cells with group *A* mutants and then examined the cells after growth at the permissive and nonpermissive temperatures for several of the classic parameters of transformation: morphology; saturation density; colony formation on plastic, on monolayers of normal cells, and in soft agar; uptake of 2-deoxy-D-glucose (2-D-G); and the expression of SV40 T and surface (S) antigens. Multiple parameters were monitored to determine whether loss of the SV40 gene *A* function at the nonpermissive temperature affected all, several, or none of the characteristics of the transformed state. Based on results from the above tests, it is concluded that the continual expression of the gene *A* function of SV40 is required for the maintenance of transformation.

MATERIALS AND METHODS

Viruses. SV40 Group *A* ts mutants *A*7, *A*28, *A*30, and *A*58, as well as the WT-2 strain, were graciously provided by Peter Tegtmeyer (29, 31). The wild-type (WT) Baylor reference strain which had been plaque-purified three times at 40.5 C was also used. Virus stocks were prepared at 33 C by inoculating confluent monolayers of CV-1 cells (obtained from Saul Kit) at a multiplicity of infection of 0.01 PFU/cell. When cytopathic effects involved 75 to 100% of the cells, the cultures were disrupted by three cycles of quick freezing and thawing, the cell lystates were clarified by low-speed centrifugation, and the supernatant fluids were stored at −70 C. Virus assays were performed in BSC-1 cells as described previously (4).

Cells. The following normal cells were employed: human skin cells derived from a patient with Fanconi anemia, obtained from P. Glade, and designated human Fanconi (HuF) cells; hamster embryo fibroblasts (HEF) prepared by trypsinizing minced embryos from Syrian hamsters (Con-Olson Co., Inc., Madison, Wisc.); and mouse BALB-3T3 cells (kindly provided by Wade Parks). In addition, the H-50 cell line, derived from a hamster tumor induced in vivo by SV40 (1, 4), and the VLM cells, derived from BALB/c mouse embryo cells transformed in vitro by SV40 and obtained from S. S. Tevethia (36), were used. With the exception of the BALB-3T3 cells, all the cell lines were grown at 37 C in Eagle medium supplemented with 10% fetal bovine serum (FBS), 100 U of penicillin and 100 μg of streptomycin per ml, and 0.075% sodium bicarbonate. The BALB-3T3 cells were grown in Dulbecco's modification of Eagle medium (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 10% FBS, 100 U of penicillin and 100 μg of streptomycin per ml, and 0.3% sodium bicarbonate at 33 C in an atmosphere of 10% CO_2. The derivation of cell lines transformed by WT and ts *A* mutants of SV40 is described below.

Saturation density experiments. Cells growing at the permissive temperature were trypsinized, and 1×10^5 to 2×10^5 cells were seeded into 35-mm plastic petri dishes. Replicate cultures were then incubated at either the permissive or the nonpermissive temperature in an atmosphere of CO_2. Cell counts were performed on duplicate samples every other day, and the media was changed every third day. A plateau in cell count was considered to represent the saturation density.

Colony formation methodology. (i) On monolayers of normal cells. Cells growing at the permissive temperature were trypsinized and plated at several concentrations, ranging between 10^2 and 10^3 cells/plate, onto confluent monolayers of human Fanconi cells in 35-mm plastic petri dishes. Cultures were incubated in 5% CO_2 at the permissive and nonpermissive temperatures, and the media were changed every third day. After 8 days at the nonpermissive temperature and 10 days at the permissive temperature, colonies were stained with Giemsa as follows: cells were washed three times with Tris-buffered saline (TBS) (pH 7.4) and fixed for 10 min in absolute methanol. The cultures were then exposed for 10 to 30 min to Giemsa staining solution which consisted of 5 ml of Giemsa stock (Matheson, Coleman, and Bell, Norwood, Ohio), 3 ml of 0.1 M citric acid, 3 ml of 0.2 M Na_2HPO_4, and 100 ml of distilled water, washed with TBS, and air dried.

(ii) On plastic. The same procedure was followed as described above, except that the cells were seeded into empty plastic petri dishes rather than onto monolayers of normal cells.

(iii) In soft agar. The technique of Macpherson and Montagnier (19) was followed. Basal layers (2 ml) of 0.5% agar (Difco) supplemented with Eagle medium, 10% FBS, and 0.2% sodium bicarbonate were formed in 35-mm plastic petri dishes, followed by the addition of a second layer (1.5 ml) of 0.3% agar supplemented as above, containing various dilutions of cells (between 10^2 and 10^3 cells/plate) which had been growing at the permissive temperature. Plates were then incubated at the permissive and the nonpermissive temperatures in atmospheres of 5% CO_2. Colony counts were performed after 7 to 10 days with a low power microscope.

Uptake of hexose. To assay the uptake of 2-D-G, cells which had been passed at least twice at either the permissive or the nonpermissive temperature were trypsinized and seeded into 35-mm plastic petri dishes (1×10^5 to 2×10^5 cells/culture). The plates were then incubated at the appropriate temperature until the monolayers were 75% confluent. At that time, the cells were washed with phosphate buffered saline (PBS; pH 7.2) at 37 C, and the plates were floated in a 37 C water bath. PBS (2 ml) containing 1 μCi of [^{3}H]2-D-G was added to each plate. After 10 min of incubation, the cells were washed with cold PBS and scraped into 1 ml of PBS, and 0.1 ml of this suspension was counted using toluene containing 4 g of 2,5-diphenyloxazole and 0.4 g of 1,4-bis-

(5-phenyloxazolyl)-benzene per liter and 10% (vol/vol) BBS-3 (Beckman Instruments, Fullerton, Calif.), in a liquid scintillation spectrometer (Model LS-250; Beckman Instruments).

Immunofluorescence techniques. Cells grown on 15-mm round cover glasses were harvested, fixed, and stained as described previously (23). SV40 T antigen was detected with sera from hamsters bearing tumors induced by SV40-transformed (H-50) cells. The hamster serum was followed by fluorescein-labeled anti-hamster baboon globulin. SV40 S antigen was detected by a method described previously (25). The S antibody was from hamsters immunized four times with PARA-adenovirus 7-transformed marmoset cells (S. S. Layne and F. Rapp, Bacteriol. Proc., p. 155, 1969).

Rescue of SV40 from transformed cells. Cellular DNA was extracted using the modified Hirt (13) procedure and passed in African green monkey kidney cells following the method described by Boyd and Butel (4). The rescued virus was passed a second time in CV-1 cells and then assayed by the plaque technique at the permissive and nonpermissive temperatures.

RESULTS

Establishment of cell lines transformed by group A mutants of SV40. Cells originating from three different species were transformed in vitro with SV40 ts mutants to compare the role of the gene *A* function in cells which are normally nonpermissive (mouse, hamster) with those which are semipermissive (human) for the replication of SV40. Cells were transformed as follows: confluent monolayers were infected with 5 to 10 PFU of virus per cell. After 90 min of adsorption at 37 C, the cells were flooded with media and incubated at the permissive temperature for 24 h, at which time they were trypsinized and subcultured. After approximately six to eight passages, transformed foci appeared in both the mouse BALB-3T3 cells and the human Fanconi cells; several passages later, those cultures were 100% SV40 T antigen positive. The normal BALB-3T3 and HuF cells exhibited a high degree of contact inhibition. This property made it easy to distinguish transformed foci from normal cells. In contrast, the HEF cells were not as highly contact inhibited and did not display such a readily distinguishable change in morphology after transformation. In addition to the lines transformed in tissue culture, newborn Syrian hamsters were inoculated with 1×10^6 PFU of SV40 ts *A*7; tumors which developed approximately 6 months later were excised and trypsinized, and the cells were established in culture.

Table 1 is a summary of pertinent information on the cell lines derived for this investigation. The designation of each cell line indicates the origin of the cells as well as the strain of SV40 used for transformation. The temperature

TABLE 1. *Origin and designation of SV40-transformed cell lines employed in this study*

Host cell origin	Strain of SV40 used for transformation	Designation of transformed cell line	Current no. of in vitro passages	Passage no. when 100% T antigen positive	Experimental temp (C)	
					Permissive	Nonpermissive
In vitro						
Mouse						
BALB-3T3	ts *A*7	Balb/*A*7	60	10	33	39.5
BALB/c embryo fibroblasts	WT-Baylor	Balb/WT-VLM	67	10	33	39.5
Hamster						
Hamster embryo fibroblasts	WT-2	Ha/WT-2	35	21	37	40.5
	ts *A*28	Ha/*A*28	30	18	37	40.5
	ts *A*58	Ha/*A*58	43	23	37	40.5
	ts *A*30	Ha/*A*30	36	22	37	40.5
Human						
Fanconi skin cells	WT-2	HuF/WT-2	20	8	37	40.5
	ts *A*28	HuF/*A*28	25	8	37	40.5
In vivo						
Syrian hamster (Con-Olsen)	WT-Baylor	H-50	>100	1	33	39.5
	ts *A*7	HaTu/*A*7-1	20	1	33	39.5
		HaTu/*A*7-2	20	1	33	39.5

selected as the permissive condition reflects the temperature at which the original normal cell line was carried. The permissive temperature for the mutant-induced hamster tumor cells and the normal BALB-3T3 cells was 33 C. The nonpermissive temperature for these two groups of cells was 39.5 C. With both the hamster and human cells transformed in vitro, it was necessary to employ 40.5 C for the nonpermissive temperature to detect phenotypic reversion; 37 C was used for permissive growth. This variation in the temperature required for nonpermissive behavior is not unprecedented. Graf and Friis (10) also found a host-range difference in nonpermissive conditions with their temperature-sensitive Rous sarcoma virus-transformed cells. It would appear that the biochemical environment within cells from different species affects the heat sensitivity of proteins to different extents. In addition, Kachani and Sabin (16) observed that hamster cells transformed in vitro by SV40 were more heat resistant than either normal hamster cells or cells from hamster tumors induced in vivo.

Morphology of transformed cells. The typical morphology of WT and group *A* mutant-transformed cells at the permissive and nonpermissive temperatures is illustrated in Fig. 1 to 3. The group *A* mutant transformed cells grown under permissive conditions form multiple layers of cells with a rounded morphology (Fig. 1C, 2C, and 3C), whereas at the nonpermissive temperature the cells are fibroblastic and highly contact inhibited (Fig. 1D, 2D, and 3D), resembling the morphology of normal cells from the same species. This difference in morphology is not easily recognizable until the cells have been passed once at the high temperature. The cells can be subcultured many passages at the nonpermissive temperature and will retain the morphology and growth characteristics of normal cells. If the cells are shifted back to the permissive temperature, they return to their original transformed morphology within 2 days. WT-transformed cells of all three species exhibit the same morphology at both temperatures (Fig. 1A, 1B; 2A, 2B; 3A, and 3B).

Saturation density. Transformed cells do not exhibit density-dependent growth control and are able to continue dividing in culture until the nutritional factors have been depleted from the media or the cells slough. As a consequence of this behavior, transformed cells achieve higher saturation densities than do normal cells which are contact inhibited. The saturation densities of the transformed cells were assayed to determine whether a decrease in saturation density accompanied the shift of mutant-transformed cells to the restrictive condition. Growth curves of the normal and transformed mouse, hamster, and human cells are shown in Fig. 4. It can be seen that WT virus-transformed cells grew more rapidly and achieved higher saturation density levels than did the corresponding normal cells from each species. The growth patterns of the WT virus-transformed cells were very similar at both permissive and nonpermissive temperatures. The ts *A* mutant-transformed cells paralleled the WT virus-transformed cells in their growth characteristics under permissive conditions. In contrast, when placed at the nonpermissive temperature, the mutant-transformed cells exhibited the slower growth rates and lower final saturation densities of normal cells. It should be noted that, although 1×10^5 to 2×10^5 cells were distributed per culture, only approximately 10 to 40% of the cells actually plated (Table 2). Therefore, the saturation density values of 2×10^5 to 4×10^5 cells per culture attained by the normal cells and by the mutant-transformed cells at the high temperature represents cell growth and not mere attachment of the cells to culture dishes.

Several saturation density experiments are summarized in Table 3. It can be seen that the maximum cell densities of all the mutant-transformed cell lines are reduced at the nonpermissive temperature. In general, the saturation densities of the mutant lines at the elevated temperature are similar to the values attained by the normal cells from which they were derived. The ratio of maximum cell density at the permissive temperature to that at the nonpermissive temperature (P/NP) averaged between 4 and 7 with most of the mutant cell lines. The tumor cell lines, HaTu/*A*7-1 and HaTu/*A*7-2, were exceptional in that they exhibited P/NP ratios of ≥ 12.0. Ratios of the saturation densities at the two temperatures with the WT virus-transformed cells (P/NP of 1.0 to 1.4) and the normal control cells (P/NP of 1.1 to 2.5) were all close to 1.

These results suggest that the inactivation of the gene *A* protein at the nonpermissive temperature causes the ts *A* mutant-transformed cells to regain the density-dependent growth controls characteristic of normal cells. It should be noted that the Balb/WT-VLM cells are not an ideal control in these studies, because they were derived from BALB/c embryo fibroblasts rather than their derivative line, BALB-3T3. Unfortunately, the BALB-3T3 cells transformed by WT virus in parallel with the Balb/*A*7 cells became

heavily contaminated with mycoplasma and were not able to be used in all experiments. However, it is pertinent that Renger and Basilico (24) compared the morphology, the colony-forming ability, and the saturation density of three lines of SV40 WT-transformed BALB-3T3 cells and found no differences in these properties at 33 and 39 C.

Colony formation under various conditions. Transformed cells have the ability to form colonies under conditions in which the growth of normal cells is inhibited, such as in soft agar or on monolayers of normal cells. Normal cells are able to plate on plastic or glass surfaces and form colonies of cells a single-layer thick; in contrast, transformed cell colonies contain multiple cell layers and stain very deeply. We examined the ability of mutant- and

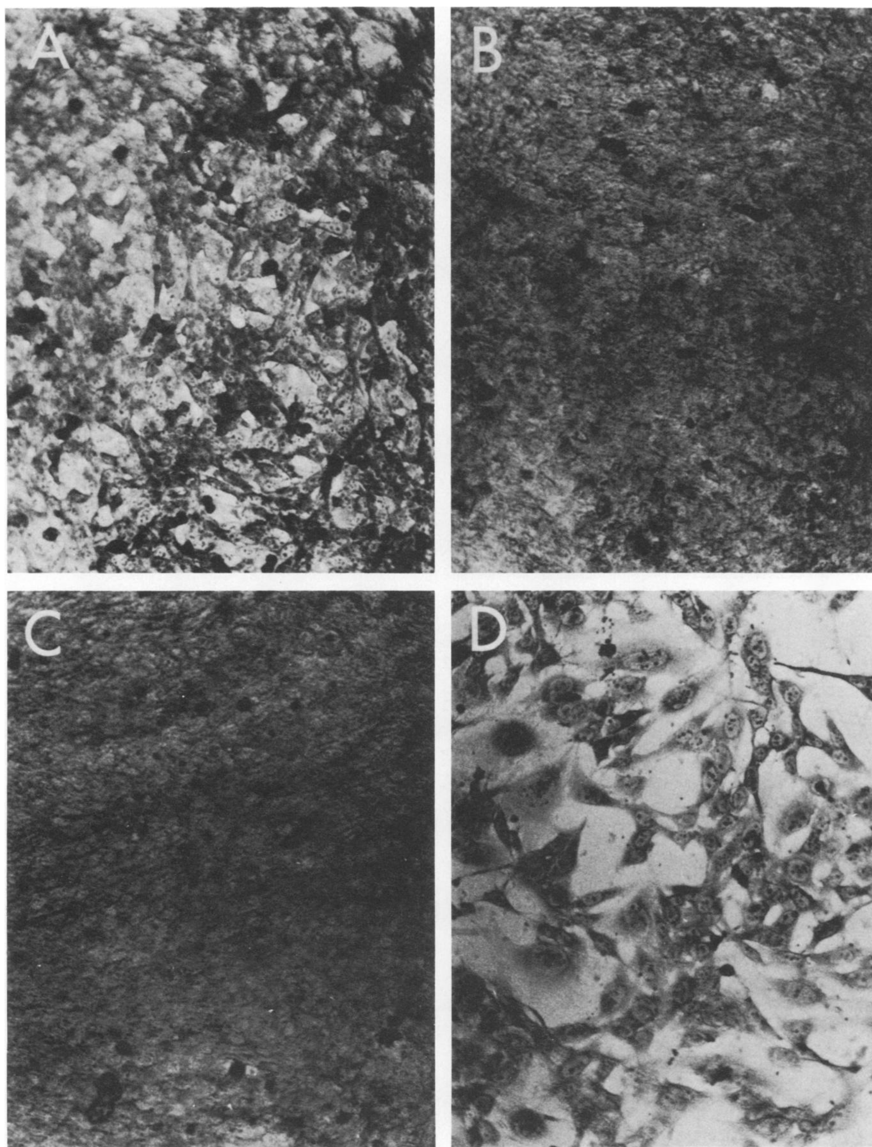

FIG. 1. *Appearance of SV40-transformed BALB-3T3 cells at the permissive and nonpermissive temperatures. (A) WT-transformed cells (Balb/WT) at the permissive temperature, 33 C. (B) Balb/WT cells at the nonpermissive temperature, 39.5 C. (C) Mutant ts A7-transformed cells (Balb/A7) at 33 C. (D) Balb/A7 cells at 39.5 C. Note the loss of transformed morphology at the nonpermissive temperature.* ×132.

WT virus-transformed cells to form colonies at the permissive and nonpermissive temperatures under these three conditions. Table 4 gives the results of colony formation on plastic surfaces. Group *A* mutant-transformed cells derived from all three species formed 4- to 51-fold fewer colonies at the nonpermissive temperature than at the permissive one. The mutant-transformed hamster and mouse cells consistently demonstrated greater temperature sensitivity than did the comparable transformed human cell line. The P/NP ratios for the former cells were all ≥ 15 (15 to 51), whereas the ratio for the mutant-transformed human cells averaged close to 4 (P/NP of 4.1 ± 1.7) from three separate experiments. WT virus-transformed cells formed similar numbers of colonies at both temperatures (P/NP of 0.8 to 1.5). In this study, only deeply stained colonies were counted to measure the number of transformed foci, not

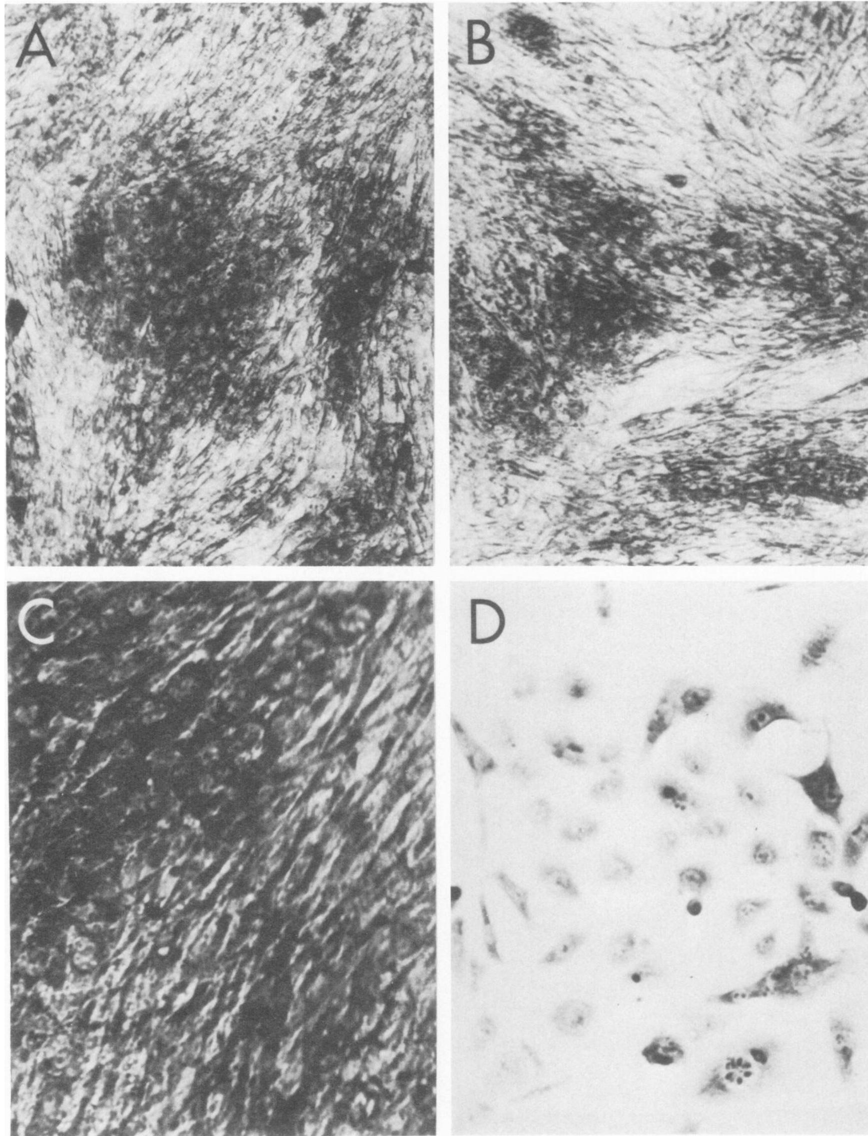

FIG. 2. *Appearance of SV40-transformed HEF cells at the permissive and nonpermissive temperatures. (A) WT-transformed cells (Ha/WT-2) at the permissive temperature, 37 C. (B) Ha/WT-2 cells at the nonpermissive temperature, 40.5 C. (C) Mutant ts A30-transformed cells (Ha/A30) at 37 C. (D) Ha/A30 cells at 40.5 C. Note the loss of transformed morphology at the nonpermissive temperature. ×132.*

the total number of normal cell colonies. Normal HEF, BALB-3T3, and HuF cells produced single-layered, very lightly stained colonies at both temperatures which were not counted in this test. Colonies were formed at the nonpermissive temperature by the mutant-transformed HEF cells; these resembled the lightly stained colonies formed by normal cells and were approximately equal in number to the transformed foci initiated by the same cells at the permissive temperature.

The observed temperature-dependent variation in colony formation could have been the result of the inability of the mutant-transformed cells to plate at the restrictive temperature. Table 2 indicates the plating efficiencies on plastic surfaces of the cell lines under study. The normal and mutant-transformed cells

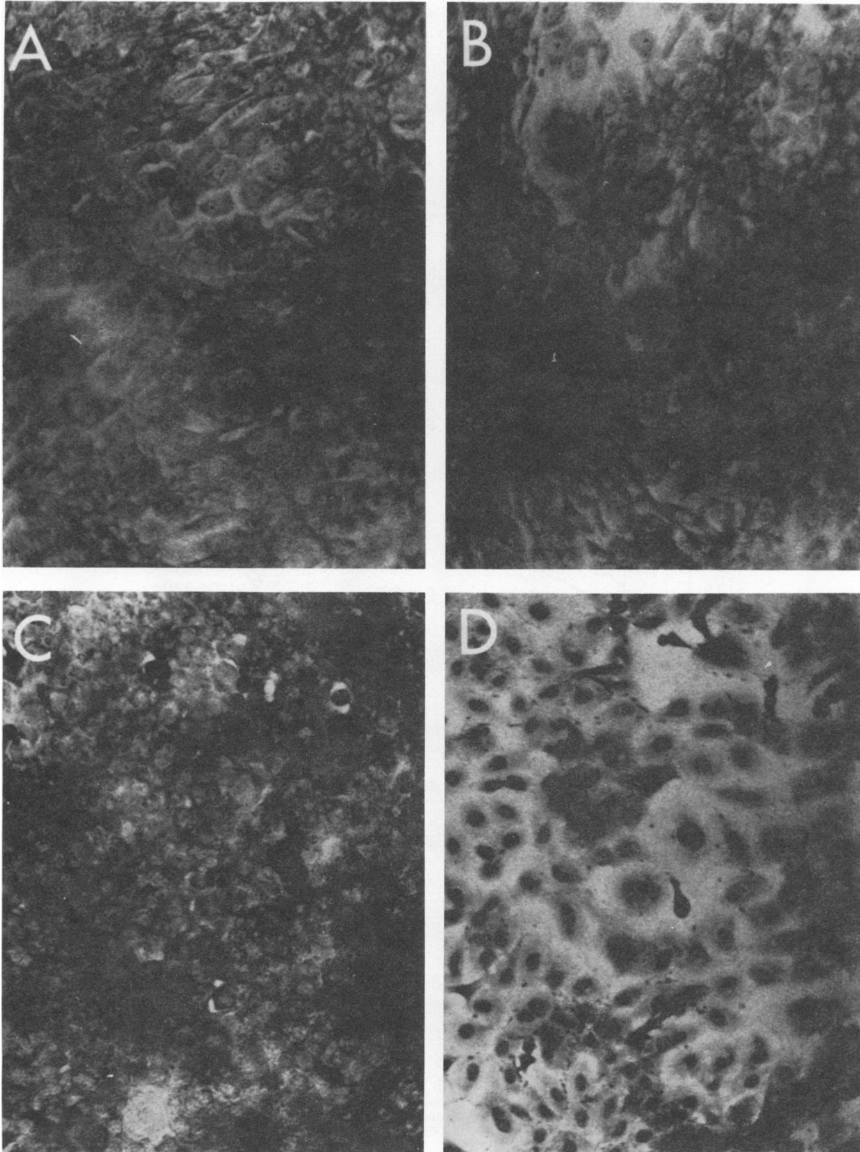

FIG. 3. *Appearance of SV40-transformed human Fanconi cells at the permissive and nonpermissive temperatures. (A) WT-transformed human Fanconi cells (HuF/WT-2) at the permissive temperature, 37 C. (B) HuF/WT-2 cells at the nonpermissive temperature, 40.5 C. (C) Mutant ts A28-transformed human Fanconi cells (HuF/A28) at 37 C. (D) HuF/A28 cells at 40.5 C. Note the loss of transformed morphology at the nonpermissive temperature. ×132.*

showed a twofold or less decreased plating efficiency at the high temperature. Therefore, it is apparent that this cannot account for the 4- to 33-fold variation in colony-forming ability described above (Table 4).

Results from experiments assaying colony formation on monolayers of normal cells (Table 5) confirm those described above. Group *A* mutant-transformed cells formed 4- to 18-fold fewer colonies at the nonpermissive temperature than at the permissive one. It should be noted that the P/NP ratios for colony formation

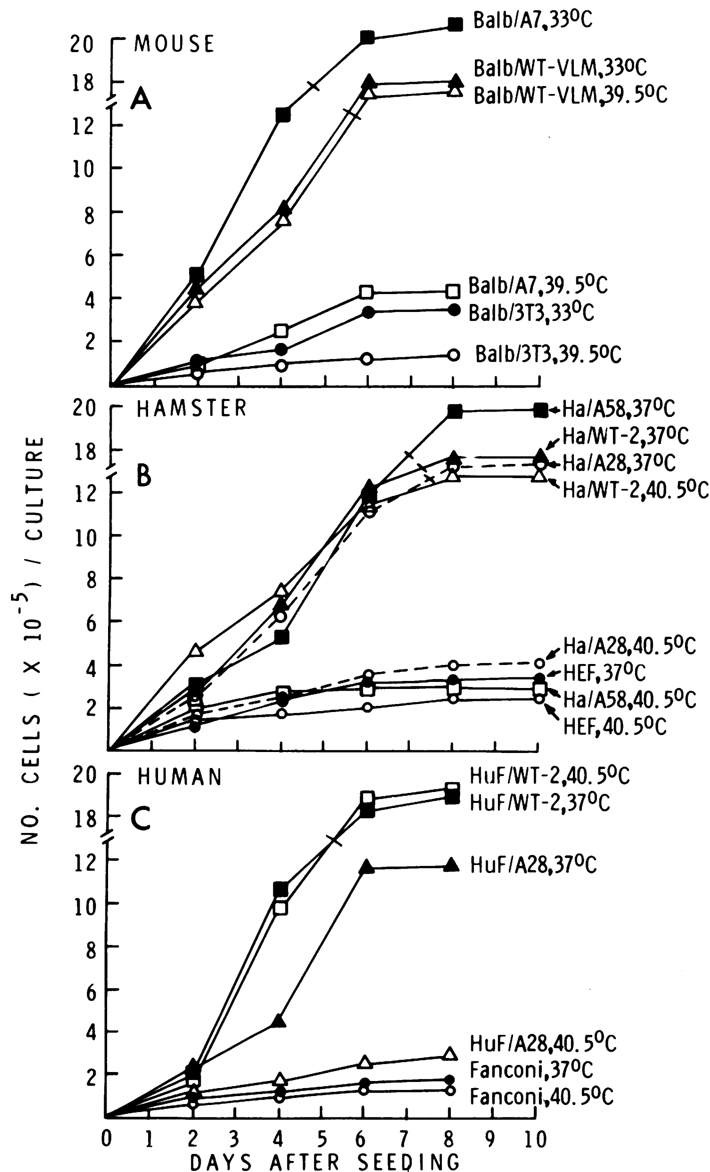

FIG. 4. *Growth curves of normal and SV40-transformed cells at permissive (33 or 37 C) and nonpermissive temperatures (39.5 or 40.5 C) were performed as described in Materials and Methods for saturation density studies. (A) Mouse cells: normal (BALB-3T3), WT-transformed (Balb/WT-VLM), and mutant ts A7-transformed (Balb/A7). (B) Hamster cells: normal (HEF), WT-transformed (Ha/WT-2), and ts A mutant-transformed (Ha/A28 and Ha/A58). (C) Human cells: normal (Fanconi), WT-transformed (HuF/WT-2), and ts A28-transformed (HuF/A28).*

on cell monolayers were lower than those found with colony formation on plastic. The frequency of colony formation (the number of colonies/10^3 cells plated) at the permissive temperature was similar under the two different conditions, so it appears that the lower P/NP ratios is a reflection of the greater ability of the transformed cells to form colonies on cell monolayers than on plastic surfaces at the high temperatures. However, single-layered colonies of normal cells could not be detected in this experiment because they were masked by the background of normal Fanconi cells. Because of this, it was not possible to determine the plating efficiencies of the transformed cell lines on monolayers of normal cells.

TABLE 2. *Plating efficiency on plastic surfaces of cells transformed by temperature-sensitive mutants of SV40*[a]

Cell line	Plating efficiency (%)		Ratio (P/NP)
	Permissive temp	Nonpermissive temp	
BALB-3T3	12	6.4	1.9
Balb/*A*7	20	12	1.7
Balb/WT-VLM	24	22	1.1
HEF	42	32	1.3
Ha/WT-2	32	30	1.1
Ha/*A*28	39	18	2.2
Ha/*A*58	41	42	1.0
Ha/*A*30	25	18	1.4
HuF	15	9.5	1.6
HuF/WT-2	32	30	1.1
HuF/*A*28	40	22	1.8

[a] Cells growing at the permissive temperature were trypsinized and seeded at various concentrations between 10^2 and 10^3 cells/plate into 35-mm plastic petri dishes containing 2 ml of media with 5% FBS. On day 3 the cells were stained and all attached single cells and small lightly stained foci were counted.

Low P/NP ratios (5.0 to 9.4) were also obtained with ts *A* mutant-transformed cells from colony formation experiments performed in soft agar (Table 6). The WT virus-transformed cells tested in soft agar (Balb/WT-VLM and H-50) formed colonies equally well at both temperatures (P/NP of 1.0 to 1.4). It is not known whether the few colonies initiated by the mutant-transformed cells at the restrictive temperature represented revertant cells or whether they were formed due to functional leak of the mutant gene *A* protein in those cells.

It is noteworthy that under all three conditions for assaying colony formation, there was a

TABLE 3. *Effect of temperature on saturation density of cells transformed by temperature-sensitive mutants of SV40*[a]

Cell line	No. of expt	Avg saturation density (no. cells × 10^5/culture)		Ratio (P/NP)
		Permissive temp	Nonpermissive temp	
BALB-3T3	2	3.5 ± 1.7	1.4 ± 0.1	2.5 ± 0.7
Balb/*A*7	4	28.0 ± 7.0	4.3 ± 1.7	7.1 ± 2.1
Balb/WT-VLM	1	17.7	17.6	1.0
HEF	1	3.7	3.4	1.1
Ha/WT-2	2	16.7 ± 3.3	13.3 ± 1.3	1.2 ± 0.05
Ha/*A*58	3	34.0 ± 0.9	6.4 ± 3.2	5.3 ± 0.7
Ha/*A*30	1	40.8	8.2	5.0
HuF	2	1.8 ± 0.3	1.7 ± 0.2	1.1 ± 0.05
HuF/WT-2	2	22.0 ± 0.4	18.0 ± 0.3	1.4 ± 0.4
HuF/*A*28	1	12.0	3.0	4.0
H-50	1	7.4	7.6	1.0
HaTu/*A*7-1	3	17.0 ± 4.0	1.5 ± 0.8	12.7 ± 5.4
HaTu/*A*7-2	2	17.0 ± 7.0	1.5 ± 0.5	12.0 ± 1.8

[a] Cells growing at the permissive temperature were trypsinized, diluted in media containing 5% FBS, and seeded into 35-mm plastic petri dishes at a density of 1×10^5 to 2×10^5 cells/plate. Replicate cultures were incubated at the permissive and nonpermissive temperatures, and cell counts were performed on duplicate samples every other day. The saturation density of a cell line is defined as that number of cells present in a culture when no rise in cell counts is seen for 3 consecutive days or longer. The results are expressed as an average of the cell counts from two or more experiments plus or minus one standard deviation. The values for the ratio, P/NP, are averages of the ratios from different experiments performed on the same cell line plus or minus one standard deviation.

TABLE 4. *Effect of temperature on colony formation on plastic surfaces by cells transformed by temperature-sensitive mutants of SV40*[a]

Cell line	No. of experiments	Colony-forming ability (no. colonies/10^3 cells plated)		Ratio (P/NP)
		Permissive temp	Nonpermissive temp	
BALB-3T3	2	0	0	
Balb/A7	1	196	10	20
Balb/WT-VLM	1	204	136	1.5
HEF	2	0	0	
Ha/WT-2	1	264	210	1.3
Ha/A28	2	124 ± 14	5.5 ± 5	23 ± 5
Ha/A58	1	180	8	22
Ha/A30	2	190 ± 34	6 ± 2	33 ± 6
HuF	1	0	0	
HuF/WT-2	1	192	244	0.8
HuF/A28	3	212 ± 34	55 ± 14.5	4.1 ± 1.7
H-50	1	250	263	1.0
HaTu/A7-1	3	236 ± 43	17 ± 11	15 ± 8.5
HaTu/A7-2	1	153	3	51

[a] Cells were plated as described in the footnote to Table 2. After 8 days at the nonpermissive temperature and 10 days at the permissive temperature, the cells were stained with Giemsa (as described in Materials and Methods), and the number of deeply stained colonies was counted. The results are expressed as an average of the colony counts plus or minus one standard deviation from different experiments performed using the same cell line. The P/NP ratios were calculated as described in the footnote to Table 3.

TABLE 5. *Effect of temperature on colony formation on cell monolayers by cells transformed by temperature-sensitive mutants of SV40*[a]

Cell line	Colony-forming ability (no. of colonies/10^3 cells plated)		Ratio (P/NP)
	Permissive temp	Nonpermissive temp	
BALB-3T3	1	1	1.0
Balb/A7	245	31	7.9
HEF	0	0	
Ha/WT-2	126	130	1.0
Ha/A28	110	6	18.3
Ha/A58	198	46	4.3
H-50	306	300	1.0
HaTu/A7-1	300	57	5.3
HaTu/A7-2	190	21	9.0

[a] Cells growing at the permissive temperature were trypsinized and seeded at concentrations ranging between 10^2 and 10^3 cells/plate onto confluent monolayers of human Fanconi cells. After 8 days of growth at the nonpermissive temperature or 10 days at the permissive temperature, the cells were stained with Giemsa (as described in Materials and Methods), and the number of deeply stained colonies was counted.

TABLE 6. *Effect of temperature on colony formation in soft agar by cells transformed by temperature-sensitive mutants of SV40*[a]

Cell line	Colony-forming ability (no. of colonies/10^3 cells plated)		Ratio (P/NP)
	Permissive temp	Nonpermissive temp	
BALB-3T3	0	0	
Balb/A7	200	30	6.7
Balb/WT-VLM	270	190	1.4
H-50	244	237	1.0
HaTu/A7-1	225	24	9.4
HaTu/A7-2	250	50	5.0

[a] Cells from the permissive temperature were trypsinized, mixed with a 0.3% agar solution, and placed over a 0.5% agar feeder layer in 35-mm plastic petri dishes. After incubation at the permissive or nonpermissive temperature for 7 to 10 days, the colonies were counted with a low-power microscope. Only those colonies containing greater than 10 cells were scored.

significant reduction in the ability of the mutant-transformed cells to form colonies at the restrictive temperature as compared to the nonrestrictive temperature. No such differences were found in the values obtained with WT virus-transformed cells at the two temperatures. These results demonstrate other growth characteristics of transformed cells which are temperature sensitive in the group *A* mutant-transformed cells.

Reversibility of phenotypic changes. To

demonstrate the reversibility of the apparent loss of the transformed phenotype with the mutant-transformed cells under restrictive conditions, a shift experiment was performed with colony formation as the assay of transformation. Normal and transformed hamster cells from the permissive temperature were seeded into plastic petri dishes, and replicate plates were incubated at 37 and 40.5 C. After incubation for 6 days, one-half of the plates at 40.5 C were shifted down to 37 C, and incubation was continued. All the plates were stained on day 11. The results are illustrated in Fig. 5. Plates seeded with normal HEF cells contained numerous single-layered, lightly stained colonies under all three temperature conditions. The WT-transformed Ha/WT-2 cells formed equivalent numbers of colonies, the majority of which were densely stained foci, under all three conditions of incubation. The mutant-transformed cells, Ha/*A*58 and Ha/*A*30, also formed numerous transformed foci at 37 C. However, when those same cells were incubated at 40.5 C for the entire 11 days, they failed to form multilayered transformed foci although many lightly stained, normal cell colonies were apparent. It is

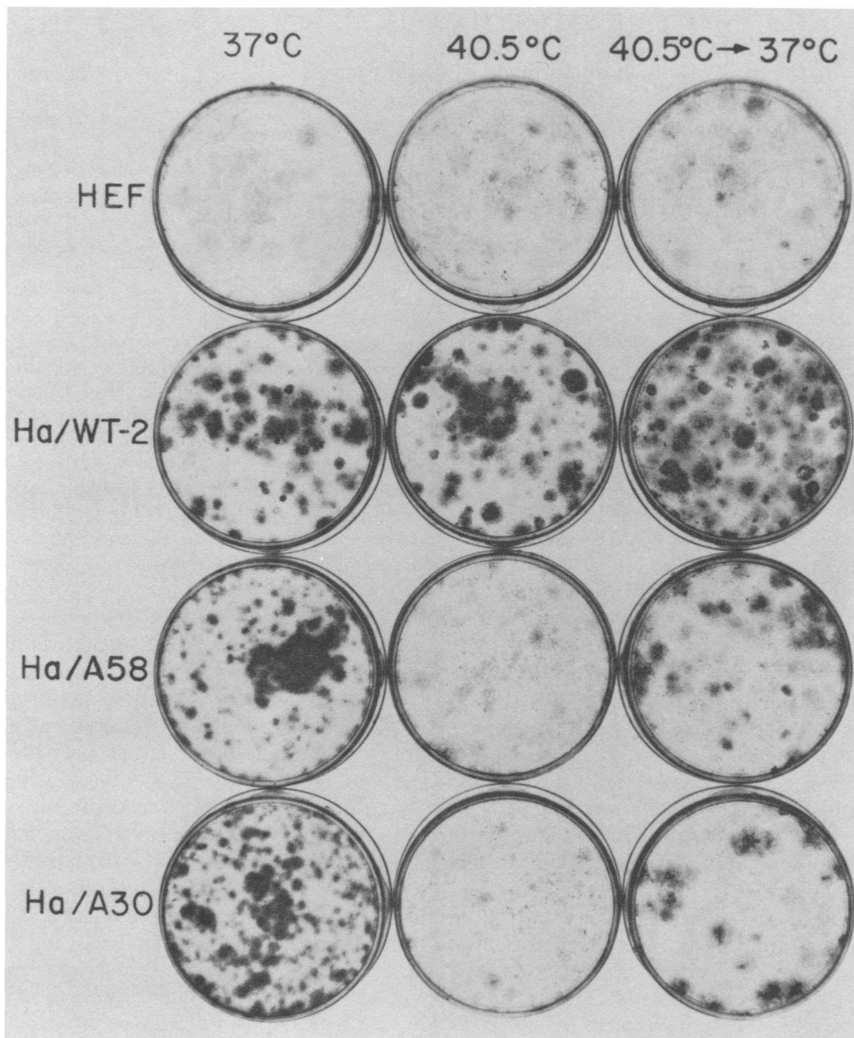

FIG. 5. *Effect of temperature shift on colony formation by SV40-transformed hamster cells. Cells were plated, and the cultures were incubated at either 37 or 40.5 C. Six days later, part of the cultures were shifted down from 40.5 to 37 C; all the plates were stained on day 11. Note the absence of transformed foci on plates seeded with mutant-transformed cells (Ha/A58 and Ha/A30) and incubated continuously at 40.5 C. Normal, lightly stained colonies were readily visible. Note also that the mutant-transformed cells, when shifted down from 40.5 to 37 C, initiated foci formation.*

significant that plates seeded with Ha/*A*58 and Ha/*A*30 cells and shifted down from 40.5 to 37 C contained many deeply stained foci. The number of colonies found on the shifted plates did not equal that formed by continuous incubation at 37 C because the plates were stained only 5 days after the shift down to 37 C.

These results indicate that the apparent reversion of ts *A* mutant-transformed cells to a normal phenotype is a reversible event when the cells are shifted back to permissive conditions. The results also suggest that the inhibition in cell growth observed with these cells at the high temperature is not due to cell death caused either by the elevated temperature or by any "toxic" effects of the mutated gene *A* protein.

Uptake of hexose. Cells transformed by either RNA or DNA tumor viruses possess an increased ability to transport certain sugars across the plasma membrane (11). Isselbacher (14) reported that SV40-transformed BALB-3T3 cells transported 2.5- to 3.5-fold greater amounts of 2-D-G than normal BALB-3T3 cells. Table 7 summarizes assays of the uptake of [^{3}H]2-D-G by normal, WT virus-, and mutant virus-transformed cells at permissive and elevated temperatures. Since the uptake of 2-D-G by normal cells has been shown to be dependent upon cell density, cultures which were 75% confluent were used for transport studies to ensure that the normal and transformed cells were in equivalent growth states. Both human and mouse cells exhibited a threefold or greater increase in uptake of 2-D-G after transformation by SV40. The transformed hamster cell lines were more variable in this property. The Ha/*A*58 cells consistently demonstrated higher levels of transport than the normal HEF cells did at the permissive temperature, but in the one experiment performed using Ha/*A*30 cells, less 2-D-G was transported by the transformed cells than by normal HEF cells. However, all the mouse and hamster cell lines transformed by group *A* mutants showed at least a threefold reduction in uptake at the restrictive temperature compared to that at the nonrestrictive one (P/NP of 3.1 to 10.7). The levels of uptake for normal cells of all three species were similar at both temperatures, as was uptake by WT virus-transformed cells (P/NP of 0.62 to 1.2). In contrast, the uptake of 2-D-G at the elevated temperature by the HuF cells transformed by ts *A*28 was actually higher than at the permissive temperature (P/NP of 0.53 to 0.83). This parameter is the only one in which the transformed human cells behaved differently from the transformed mouse and hamster cells. Thus, sugar uptake demonstrates another property which is temperature-sensitive in most group *A* mutant-transformed cells.

TABLE 7. *Effect of temperature on uptake of [^{3}H]2-D-G by cells transformed by temperature-sensitive mutants of SV40*[a]

Cell line	Expt no.	Uptake of 2-deoxyglucose (counts per min/10^5 cells)		Ratios (P/NP)
		Permissive temp	Nonpermissive temp	
BALB-3T3	1	681	730	0.9
Balb/*A*7	1	4,550	488	9.3
	2	10,323	961	10.7
	3	10,093	2,148	4.7
Balb/WT-VLM	1	2,900	2,610	1.1
HEF	1	3,067	3,661	0.84
	2	1,324	1,058	1.2
Ha/WT-2	1	6,910	11,098	0.62
Ha/*A*58	1	6,557	1,320	5.0
	2	5,409	1,730	3.1
Ha/*A*30	1	1,207	216	5.6
HuF	1	250	330	0.76
HuF/*A*28	1	761	1,426	0.53
	2	1,935	2,330	0.83
HuF/WT	1	2,908	2,626	1.1

[a] Petri dishes (35 mm) containing 75% confluent monolayers of cells were washed with PBS (pH 7.2) and warmed to 37 C. After the addition of 2 ml of PBS containing 1 μCi of [^{3}H]2-D-G per ml, the cells were floated in a 37 C water bath. After 10 min, the cells were washed with cold PBS and scraped into 1 ml of PBS, and 0.1 ml of this suspension was assayed for radioactivity (see Materials and Methods). Triplicate samples were counted, and the results are expressed as an average of the three cultures. Cell counts were determined on replicate cultures which were incubated under identical conditions as those assayed for uptake of hexose.

Expression of SV40-induced antigens. The group *A* protein has not yet been definitively correlated with any of the SV40-specific antigens found in transformed cells. Since there is only enough SV40-specific RNA in most transformed cells to code for 1 to 3 proteins, it is likely that the gene *A* product is at least one of these antigens. Table 8 contains the results from immunofluorescence tests for two antigens, T and S, in the mutant-transformed cells. In tests for T antigen, no differences were found in the number of antigen-positive cells or the degree of fluorescence in cells grown at the two temperatures. In contrast, S antigen was not detected in cultures of mutant-transformed cells grown at the nonpermissive temperature. Since the nature and identity of S antigen

TABLE 8. *Effect of temperature on synthesis of virus-induced antigens in cells transformed by temperature-sensitive mutants of SV40*

Cell line	T antigen[a]		S antigen[b]	
	Permissive temp	Nonpermissive temp	Permissive temp	Nonpermissive temp
Balb/WT-VLM	+[c]	+	+	+
Balb/*A*7	+	+	+	0
Ha/WT-2	+	+	+	+
Ha/*A*58	+	+	+	0
H-50	+	+	+	+
HaTu/*A*7-1	+	+	+	0
HuF/WT	+	+	+	+
HuF/*A*28	+	+	+	0

[a] T (tumor) antiserum was prepared by inoculating weanling Syrian hamsters with SV40-transformed hamster cells. Animals were bled after the appearance of tumors. Cells grown at either the permissive or nonpermissive temperature were fixed for 3 min with acetone and stained by the indirect immunofluorescence technique as described (23).

[b] S (surface) antiserum was prepared by immunizing hamsters four times with mycoplasma-free PARA-transformed marmoset cells. Unfixed cells were stained by an indirect immunofluorescence technique described previously (25). The reaction of the S antiserum with SV40-transformed cells was not diminished by absorption with either sheep red blood cells or herpes simplex virus type II-transformed hamster cells.

[c] +, Positive reaction; 0, negative reaction with immunofluorescence reagents.

remains obscure, it is difficult to interpret these results. The anti-S sera used in this study appear to react with an SV40-specific antigen(s) (see footnote to Table 8), but Häyry and Defendi (12) prepared a similar reagent which was able to react with normal cell antigens uncovered by trypsin treatment. Therefore, until more is known concerning the nature of this antigen, it can only be concluded that there is a strong correlation between the presence of S antigen and SV40 transformation. Thus, these results indicate another phenotypic property of SV40 transformation which is temperature-dependent in cells transformed by group *A* mutants.

Rescue of transforming virus. It is critical to establish that the observed temperature-dependent phenotypic expressions of transformation in the group *A* mutant-transformed cells is due to a mutant viral function and is not a reflection of an altered cellular function. Using a special selection procedure, Renger and Basilico (24) isolated an SV40-transformed cell line with properties similar to our group *A* mutant-transformed lines. However, when virus was rescued from the former cells, it was found to be WT in its growth properties. This indicated to the authors that the fluctuation in the expression of the transformed state was due to a mutant cellular function. Since no selective conditions were applied during the derivation of our cell lines and since they were not cloned, it seems highly unlikely that only ts cells would have been transformed and selected in the evolution of all the transformed cell lines of all three species, and then only from those cultures exposed to ts mutant virus. However, to characterize the transforming virus, SV40 was rescued from representative ts *A*-transformed cell lines of each species by the DNA transfer method (4), and the rescued virus was assayed at the permissive and nonpermissive temperatures to determine if it retained its temperature sensitivity (Table 9). The virus rescued from group *A* mutant-transformed mouse, hamster, and human cells was, in fact, temperature sensitive. The relative plating efficiency at 40.5 compared to 33 C ranged from 6.2×10^{-3} to 1.3×10^{-2}. Although it cannot be proved that the viruses recovered in this experiment represent the genomes responsible for the transformation events, these results suggest that the temperature-dependent properties of the group *A* mutant-transformed cells are due, in all probability, to a defective viral gene product and not an altered cellular function.

TABLE 9. *Temperature-sensitive nature of virus rescued from SV40 group A mutant-transformed cells*[a]

Cell line from which virus was rescued	Titer of rescued virus (PFU/ml)		Efficiency of plating[b]
	40.5 C[c]	33 C[c]	
Balb/*A*7	$<1.0 \times 10^2$	3.0×10^4	$<3.3 \times 10^{-3}$
HaTu/*A*7-1	$<1.0 \times 10^2$	4.7×10^4	$<2.1 \times 10^{-3}$
Ha/*A*28	1.1×10^3	8.5×10^4	1.3×10^{-2}
Ha/*A*58	2.3×10^1	3.7×10^3	6.2×10^{-3}
HuF/*A*28	$<1.0 \times 10^2$	4.0×10^4	$<2.5 \times 10^{-3}$

[a] Virus was rescued from the transformed cells by transfection by the procedure described in Materials and Methods. The rescued virus, which had been passed once through African green monkey kidney cells and once through CV-1 cells, was then assayed by the plaque technique (see Materials and Methods) at the permissive (33 C) and nonpermissive (40.5 C) temperatures. Under these conditions, WT SV40 has an efficiency of plating (40.5 C/33 C) of 1.0. The ts *A* mutant stocks have efficiency of plating values which range from 10^{-3} to 10^{-6}.

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

[c] Assay temperature.

DISCUSSION

This paper has presented evidence which suggests that cells transformed by SV40 ts mutants from complementation group *A* exhibit the phenotypic characteristics of transformed cells when grown at the permissive temperature and those of normal cells when cultured under nonpermissive conditions. Three different types of parameters were examined to monitor transformation: (i) those based on the degree of contact inhibition of the cells (saturation density and colony formation), (ii) those based on enzyme function in the plasma membrane (uptake of 2-D-G), and (iii) those based on the synthesis of SV40 antigens. The expression of all of the above criteria of transformation was found to be temperature sensitive. Therefore, it is concluded that the SV40 gene *A* protein is essential for the maintenance of growth characteristics generally accepted to be typical of transformation.

Since the apparent reversion to a normal phenotype of the ts *A* mutant-transformed cells at the elevated temperature was monitored primarily on the basis of parameters which measure cell growth, it might be speculated that the observed phenomena were merely a reflection of cell stasis or death at the high temperature. Several observations suggest this explanation is incorrect and indicate, rather, that the cells are undergoing phenotypic reversion. (i) WT virus-transformed cells did not exhibit any temperature sensitivity at the restrictive temperatures used in these experiments, indicating that the temperatures selected for study could be tolerated by transformed cells. (ii) The growth characteristics of the ts mutant-transformed cells at the elevated temperature were very similar to those of normal cells derived from the same species (Fig. 4), showing that the cells were able to divide at the high temperature. (iii) All of the mutant-transformed cell lines, with the exception of the tumor cell lines, could be passed repeatedly at the nonpermissive temperature. The cells maintained the morphology and contact-inhibited growth behavior of normal cells throughout the passages (Fig. 1D, 2D, and 3D). If the cells were unable to grow under restrictive conditions, such continual passage would not be possible. (iv) The mutant-transformed cells were able to produce single-layered colonies at the nonpermissive temperature which stained lightly and resembled normal cell colonies (Fig. 5). (v) The shift-down experiment (Fig. 5) indicated that cell death and/or toxicity of a mutant gene product does not account for the inability of the mutant-transformed cells to form typical transformed foci at the high temperature. If either of those suggestions were correct, the cells would be unable to initiate new growth and would not develop foci when shifted down to the permissive temperature. (vi) The cells were able to actively transport 2-D-G at the nonpermissive temperature (Table 7), again showing the viable state of the mutant-transformed cells. (vii) The preceding evidence is strengthened by the cell counts performed in the saturation density experiments. The cell counts were performed with a vital dye, trypan blue, which readily differentiates the living from the dead cells; only the viable cells were counted, and these increased in number throughout the experiments.

The fact that multiple properties of the cells fluctuated in unison suggests that the gene *A* function is involved in the primary events controlling the expression of the transformed state. Mutant-transformed cells of mouse, hamster, and human origin behaved similarly, indicating that the gene *A* protein interacts in the same manner with constituents of cells which are both nonpermissive and semipermissive for SV40 replication. The only lack of similarity observed in the phenotypic properties of the transformed cells from the three different species was the failure of the HuF/*A*28 cells to exhibit a temperature-sensitive uptake of 2-D-G. The significance of this finding remains to be elucidated, but, with this one human cell line, it appears that increased transport of hexose can be separated from the other phenotypic expressions of transformation. Studies are in progress to define the role of the gene *A* function in transformed monkey cells to determine whether a similar temperature-sensitive expression of transformation is seen in cells from a permissive species following transformation by SV40 ts *A* mutants.

The group *A* mutants were originally isolated on the basis of loss of infectivity at the elevated temperature (31). Therefore, the evidence presented in this paper and the accompanying reports (21, 22, 30) reveal that in the SV40 system at least one function is required both for replication of intact virus and for maintenance of transformation. Since the SV40 genome contains only a limited amount of genetic information, it is not surprising that a virus-specific function would serve such a dual role. However, this situation is apparently not always the case with RNA tumor viruses. In the avian system, transformation functions can be separated from replication functions. Temperature-sensitive mutants have been isolated which are defective

only for replication (33), defective only for transformation (3, 20), or defective for both (35).

During SV40 lytic infection, the gene *A* product has been found to be directly responsible for the initiation of viral DNA synthesis (29). However, the function is not essential for the continuation of viral DNA replication once it has been initiated. It appears, therefore, that SV40 carries its own control system, or replicon, for the regulation of viral DNA synthesis. This information provides a clue concerning the possible function of the gene *A* product in transformed cells. It is possible that normal cellular DNA synthesis comes under the control of the viral initiator function in transformed cells. This could be achieved by the viral initiator protein interacting with cellular operator regions to stimulate cellular DNA synthesis. Alternatively, the mere initiation of replication of the integrated viral genome at the viral initiator site could trigger a complete round of cellular DNA synthesis. Either mechanism could cause normal cellular control processes to be superseded by those of the integrated virus. In the absence of normal regulatory controls, cellular DNA synthesis could be triggered repetitively by a continuously expressed gene *A* function. This model is described in more detail elsewhere (J. S. Butel, J. S. Brugge, and C. A. Noonan, Cold Spring Harbor Symp. Quant. Biol., in press). Models with similar features have also been proposed recently by others (R. G. Martin, J. Y. Chou, J. Avila, and R. Saral, Cold Spring Harbor Symp. Quant. Biol., in press; 18, 29).

The fact that ts *A*-transformed cells revert to a normal phenotype at the elevated temperature is probably due to the heat inactivation of the gene *A* protein. With the loss of functional viral initiator protein, normal regulatory mechanisms resume control of DNA replication. The return to normal patterns of host cell DNA synthesis should restore some, if not all, of the phenotypic properties of normal cells. The loss of the transformed phenotype observed at the nonpermissive temperature is a fully reversible phenomenon; the transformed characteristics reappear after the cells are shifted back to the permissive temperature. This suggests that the transformed phenotype is controlled by a product of the SV40 genome and that the viral DNA itself is not excised or lost from the cells when reversion occurs.

The nature of the viral initiator protein is not known. It is possible that this protein could function as an endonuclease since an endonucleolytic cut in superhelical SV40 DNA would be essential for the initiation of viral DNA synthesis. Virions of a similar mutant of polyoma virus, TS-a (also known to be defective in viral DNA synthesis), were found to carry a defective endonuclease activity (6). In addition, Ritzi and Levine (26) found that SV40 group *A* mutants cause less fragmentation of cellular DNA than WT virus during lytic infection at the nonpermissive temperature. This evidence also suggests a defective endonuclease activity.

Although the synthesis of T antigen does not appear to be temperature-sensitive in group *A* mutant-transformed or infected cells, the possibility that the gene *A* protein is actually T antigen cannot be ruled out. A single amino acid change in the active site of a protein would not necessarily affect the integrity of its antigenic determinants. The sedimentation properties of T antigen from ts *A*-infected cells incubated at permissive or nonpermissive temperatures have been found to be different (M. Osborn and K. Weber, personal communication). This suggests that while the antigenic site of the protein is not affected by temperature, the total configuration of the polypeptide is altered enough to cause a change in its S value. It should also be remembered that all SV40-infected and transformed cells possess T antigen (2, 5), suggesting that its function, like that of the gene *A* protein, is essential both for SV40 replication and for maintenance of transformation.

It would be informative to know the status of the tumor-specific transplantation antigen (5) in the SV40 ts *A*-transformed cells. If its presence were also observed to fluctuate when the cells were shifted from permissive to nonpermissive conditions, that would constitute suggestive evidence that tumor-specific transplantation antigen is also under the control of SV40 gene *A*.

A single size class of early SV40 mRNA which sediments at 19*S* has been found in productively infected cells (34). This observation suggests that the 19*S* RNA molecule might be translated into a single, large polypeptide which might, subsequently, be cleaved into smaller polypeptides in a manner analogous to the pattern of cleavages which occurs in the poliovirus system (15, 28). Either the large primary translation product or the cleavage products may possess multiple functions in the cell (i.e., stimulation of host cell DNA synthesis, initiation of viral DNA synthesis, helper activity for adenovirus replication, etc.). If a lesion in one part of the gene affected the processing of the primary polypeptide, the other functions would be affected as well. For example, the viral

initiator function might be located on a different cleavage product from the function responsible for the induction of cell DNA synthesis, but both activities would appear to be inactivated under nonpermissive conditions. It is not possible on the basis of available data to distinguish in the SV40 system the situation of a precursor polypeptide which gets cleaved from the presence of a single multifunctional early protein.

One previous theory of the mechanism of virus-mediated transformation hypothesized that the mere integration of a foreign viral genome into a cellular chromosome was sufficient to cause transformation (37). The identification of a viral function (the gene *A* protein) which is essential for the maintenance of SV40-induced transformation proves that integration alone is not sufficient for stable transformation, but, rather, that the continual expression of at least one viral protein is required. This fact also suggests that the DNA tumor viruses are acting directly on the cell during transformation and not indirectly by the activation of an RNA tumor virus as proposed by the oncogene hypothesis (32). This observation does not rule out the possibility, of course, that DNA and RNA tumor viruses might interact under appropriate conditions and might even exhibit an enhanced transforming ability under conditions of joint infection.

The only previous suggestion of viral control of the transformed phenotype in a DNA tumor virus system was with BHK-21 cells transformed by polyoma virus mutant ts-3 (7, 8). With those cells, only two parameters of transformation (topoinhibition and wheat germ agglutination) reverted to normal when the cells were elevated to the nonpermissive temperature. Two other properties of the cells (growth in soft agar and wound serum requirement) remained transformed. Another group of polyoma ts mutants, typified by TS-a, appear to resemble SV40 group *A* mutants during lytic infection (9). However, experiments performed to date with the polyoma virus ts *A* mutants have led to the conclusion that the function is required only for the initiation and not the maintenance of transformation. It is possible that the polyoma virus ts *A* function differs somewhat from the ts *A* function of SV40. Alternatively, experiments designed to examine additional parameters of polyoma ts *A* mutant-transformed cells (other than the soft agar assay at 38.5 C) might detect a similar role for that viral protein in the maintenance of the transformed state.

It has been reported previously that SV40 gene *A* product is essential for the initiation of transformation (17, 29). In those studies, cells were infected and plated at the nonpermissive temperature, and the number of colonies was counted about 30 days later. The present finding that the gene *A* function is required for the maintenance of transformation makes the interpretation of those results more difficult, if initiation is considered to be that initial event which commits a normal cell to ultimately express the characteristics of a transformed cell. Since the production of a focus requires multiple cell divisions, it would appear that the maintenance of transformation is required for a focus to be formed. To assay the initiation of transformation at the nonpermissive temperature, it would be necessary to infect cells, incubate them for a limited time at the nonpermissive temperature, and then shift them to permissive conditions to allow foci to form, if they had been initiated under restrictive conditions. If ts *A*-infected cells were left under nonpermissive conditions for the entirety of an experiment, foci could not develop even if initiated, because the transformed state would not be maintained. Because of these reservations about the interpretation of those data, it is not possible to conclude yet whether another SV40 function exists for the initiation of transformation, or whether the gene *A* protein accomplishes both steps in the transformation process.

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