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Function of Simian Virus 40 Gene A in Transforming Infection

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In productive infection by simian virus 40, the *A* gene is known to regulate the initiation of viral DNA replication and to control the synthesis of late viral RNA. The function of the *A* gene in transforming infection was investigated by the infection of a variety of cell species with six independently isolated temperature-sensitive mutants belonging to the *A* complementation group. The *A* mutants failed to initiate the stable transformation of cells during continuous infection at the restrictive temperature. After the establishment of transformation at the permissive temperature and a subsequent shift to the restrictive temperature to block the *A* function, however, two distinct virus-cell interactions were identified. In one case, the increased colony-forming capacity of transformed cells remained stable after the temperature shift. In the other case, the temperature shift decreased the capacity of transformed cells to form colonies to the level of untransformed control cells. The outcome of the virus-cell interaction depended both on the nature of the *A* mutation in a given cell species and on the species of the cell transformed by a given mutant. These findings suggest that the transformation process may require two distinct events, each related to *A* gene expression.

Previous reports have shown that the temperature-sensitive *A* mutants of polyoma (5, 6, 8) and simian virus 40 (SV40) (9, 14) are unable to establish the stable transformation of certain cell lines under restrictive conditions. In several of these studies, however, the transformed phenotype was no longer temperature-sensitive once the cells were stably transformed under permissive conditions (5, 6, 8, 9). The conclusion that the *A* gene of each virus is required to initiate, but not to maintain, the transformed state has been widely accepted, even though a limited number of viral mutants and transformed cell lines were examined.

Studies of *A* function in the productive infection of permissive monkey kidney cells have shown that the *A* protein continuously regulates the initiation of viral DNA replication (3, 14) and transiently controls the synthesis (4) of late viral RNA. It seemed quite possible that the *A* protein could also affect cellular DNA replication or transcription under appropriate circumstances and thereby influence the growth of transformed host cells. Thus, a more extensive study of the effect of gene *A* on the growth characteristics of transformed cells was undertaken with a number of well-characterized *A* mutants in a variety of host cells. Cellular growth was quantitated exclusively by a simple colony assay which was found to be quite sensitive and reproducible.

This study showed that the expression of gene *A* may indeed have a marked effect on the efficiency of colony formation of cells transformed by SV40. Furthermore, when considered in complete perspective, the present findings are consistent with the data previously reported by others (5, 6, 8, 9). It would be premature, however, to assume that the function of the SV40 *A* gene is directly required to maintain the transformed state of cells.

MATERIALS AND METHODS

Cell cultures. The TC7 clone (13) of the CV-1 line of monkey kidney cells, Swiss 3T3 cells (17), rabbit kidney cells (Flow Laboratories), and Syrian hamster cells (Flow Laboratories) was cultivated in Eagle medium containing 5 to 10% fetal bovine serum. The rabbit and hamster cells were inoculated with virus after two to four passages in culture. The permissive temperature for the temperature-sensitive viral mutants was 33 C in each cell line, but the restrictive temperature was determined by the viability of each cell line at elevated temperatures.

Virus. The *A* and *B* groups of mutants were derived from a single clone of wild-type (WT) virus after mutagenesis with nitrosoguanidine or hydroxylamine as previously described (14, 15). All of the *B* mutants produce infectious viral DNA, but not infectious virus, at the restrictive temperature (16), whereas the *A* mutants are defective in viral DNA replication (14). Virus stocks were grown at 33 C after inoculation of TC7 monolayers with 0.01 PFU/cell. Virions were assayed at 33 and 40 C as previously described (15).

T antigen assay. The presence of SV40 T antigen in transformed cells was demonstrated by the indirect immunofluorescence technique with serum from hamsters bearing virus-free, transplanted tumors and rabbit, fluorescein-conjugated, anti-hamster immunoglobulin G by the method of Pope and Rowe (12).

Assay for the initiation of transformation. Subconfluent cultures of cells were inoculated with virus stocks containing 10^8 PFU/ml. After adsorption for 2 h at room temperature, the inoculum was replaced with fresh medium. Duplicate cultures were incubated at the permissive temperature for 3 to 4 weeks and at the restrictive temperature for 2 to 3 weeks. The cultures were then washed with Tris-buffered saline, fixed with isopropyl alcohol, and stained with Coomassie blue in 10% acetic acid. Colonial morphology was used to identify transformed clones. Reconstruction experiments with transformed cells showed that secondary seeding of colonies from primary transformants was minimal under these conditions. The frequency of transformation of rabbit and hamster cells was less than 0.1%.

Assay for the temperature dependence of the colony-forming characteristics of cells after stable transformation. Subconfluent cells were inoculated with 10^8 PFU/ml. After adsorption for 2 h at room temperature, the inoculum was replaced with medium. The cells were subcultured 6 to 10 times at 33 C until all of the cells appeared transformed and contained T antigen. The cells were not cloned, so that heterogeneous populations of transformed cells were examined. The only selective pressure on the cells was a selection for rapid growth at 33 C. Control nontransformed cells or uniformly transformed cells were dispersed with 0.05% trypsin and serially diluted in medium. Equal portions of each cell dilution were incubated for 3 weeks at the permissive temperature and for 2 weeks at the restrictive temperatures. Colonies were fixed, stained, and quantitated as described above.

TABLE 1. *Temperature-sensitive characteristics of transformation of 3T3 cells and rabbit kidney cells by WT virus and mutants of SV40*

Transforming virus	Transformed colonies (permissive temp/restrictive temp)			
	3T3 cells[a]		RK cells[b]	
	Initiation of transformation[c]	Colony formation after transformation[d]	Initiation of transformation	Colony formation after transformation
WT	1	6	2	1
B4	1	3	NT[e]	1
*A*28	156	7	68	82
*A*30	119	8	227	3

[a] 3T3 cells were tested at 33 and 39 C.

[b] RK cells (rabbit kidney) were tested at 33 and 41.5 C.

[c] The initiation of transformation was quantitated by exposing untransformed cells to virus during continuous incubation at either the permissive or restrictive temperature. The temperature dependence of initiation was determined by the ratio of the number of transformed colonies at 33 C/number of transformed colonies formed at the restrictive temperature.

[d] Colony formation by cells after the establishment of stable transformation at 33 C was quantitated by serially diluting and culturing suspensions of single cells at either the permissive or restrictive temperature. The temperature dependence of colony formation was determined by the ratio of the number of colonies at 33 C/number of colonies at the restrictive temperature. Essentially no contact-inhibited colonies were formed at either temperature.

[e] NT, Not tested.

RESULTS

Transformation of 3T3 cells. The transformation of 3T3 cells was investigated because this contact-inhibited cell line has been well characterized and is efficiently transformed by SV40 (1, 17). Unfortunately, 3T3 cells are destroyed by prolonged exposure to temperatures over 39 C, and most of the *A* mutants are relatively leaky at 39 C (14). Nevertheless, previous studies had shown that the *A* mutants are defective in the initiation of stable transformation at 39 C (14). These studies were extended to determine the effect of growth at 39 C on the stability of the transformed phenotype after establishment at 33 C as determined by the colony assay described above (Table 1). When 3T3 cells were first stably transformed by *A*28 or *A*30 at 33 C and then shifted to 39 C as dilute suspensions of single cells, the cells formed colonies with a transformed phenotype as well as cells transformed by WT virus. Each of the transformed cell lines was slightly temperature-sensitive at 39 C. Essentially all of the cells in the heterogeneous population tested in these studies must have been transformed because no contact-inhibited colonies grew at either 33 or 39 C.

Transformation of rabbit kidney cells. The transformation of rabbit cells was investigated because the cells grow well at 41.5 C. Most of the mutants have little if any detectable function in the productive infection of monkey cells at this temperature (14). Further, nontransformed rabbit cells rarely undergo "spontaneous" transformation and have a very low efficiency of colony formation in comparison to cells transformed by SV40. Thus a temperature dependence of the capacity of transformed rabbit cells to form colonies would be expected to be easily detectable.

Table 1 compares the ability of the mutants to initiate the transformation of rabbit cells during continuous incubation at 41.5 C and to

retain the colony-forming characteristics of transformed rabbit cells after a shift to the restrictive temperature. The *A* mutants were unable to establish transformation of rabbit cells at 41.5 C. Cells which had been transformed at 33 C were rapidly subcultured until all of the cells contained T antigen and the cultures appeared to be uniformly transformed. Uninfected control cells could not be successfully grown under the same conditions of subculture. When the transformed cells were dispersed, diluted, and assayed for growth at either 33 or 41.5 C, cells transformed by WT virus and five of the six *A* mutants produced transformed colonies with approximately the same frequency at both temperatures. Only cells transformed by *A*28 were significantly reduced in colony-forming efficiency at the higher temperature. Nevertheless, essentially all of the *A*28 colonies which did grow at higher cell concentrations had the appearance of dense transformed colonies. Similar results were obtained with two additional rabbit cell lines independently transformed by *A*28. None of the cells in the transformed cultures produced contact-inhibited colonies at either temperature. Thus the expression of the *A* gene seemed to affect the growth characteristics of rabbit cells stably transformed by *A*28 but not cells transformed by the other *A* mutants. These findings suggest that the nature of the defect in the *A* protein may influence its effect on any given host cell.

Transformation of hamster embryo cells. Syrian hamster cells were studied because they can be grown at relatively high temperatures (40 to 40.5 C) and can be continuously cultivated without apparent spontaneous transformation. The initiation of transformation was difficult to quantitate using hamster cells inasmuch as transformation was inefficient and required long time intervals. Further, uninfected cells tended to grow in crowded foci to a limited extent under the conditions of the assay procedure. Figures 1 and 2 show the behavior of hamster cells at 33 and 40 C after the establishment of stable transformation at 33 C. Control, nontransformed hamster cells grew at high cell densities at 40 C but were quite temperature sensitive in colony formation at low cell densities. Transformation by WT virus or late SV40 mutants efficiently overcame this density dependence of cell growth at 40 C. In contrast, cells transformed by all of the *A* mutants except *A*57 produced 10- to 1,000-fold fewer colonies at 40 than at 33 C and thus resembled nontransformed cells in this growth property. However, at higher cell concentrations at 40 C, hamster cells transformed by all of the *A* mutants formed dense colonies with the typical appearance of transformed colonies (Fig. 3). No contact-inhibited colonies were formed at any cell density at either temperature by the transformed cells.

Microscopic examination of cultures which contained no colonies of cells transformed by the *A* mutants revealed that individual cells had attached to the surface of the culture vessel (Fig. 4). The cells were flat and each contained a single nucleus. Some of these cells were so large that they were macroscopically visible on close examination. These findings suggested that some cells were viable but unable to undergo cell division. To test the reversibility of this growth inhibition, cultures containing single cells, unable to grow after 2 weeks of incubation at 41 C, were shifted back to 33 C

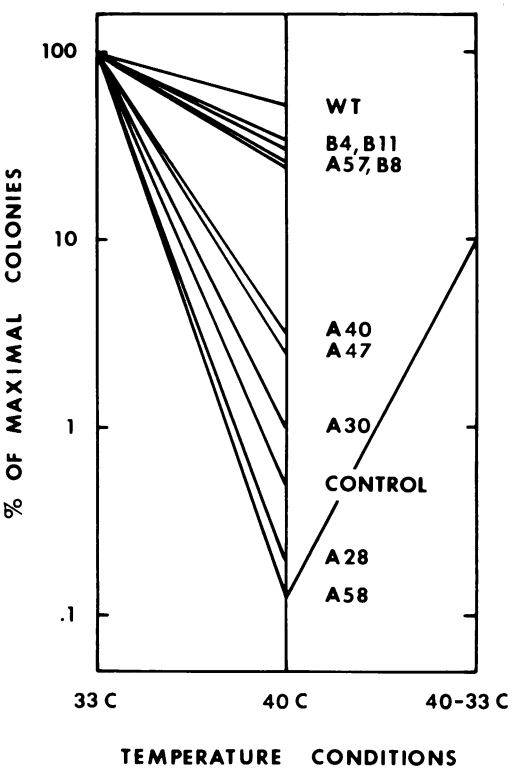

FIG. 1. *Efficiency of colony formation by hamster embryo cells after the initiation of stable transformation at 33 C. Uninfected cells and cells transformed by WT or mutant virus were dispersed, serially diluted, and cultured at 33 or 40 C. After 2 to 3 weeks the cultures were fixed and stained, and colonies were counted. In some cases, duplicate cultures were shifted from 40 C after 2 weeks and incubated 2 more weeks at 33 C before assay of colony formation. The transforming virus for each cell line is designated in the figure. The appearance of selected samples is shown in Fig. 2 and 3.*

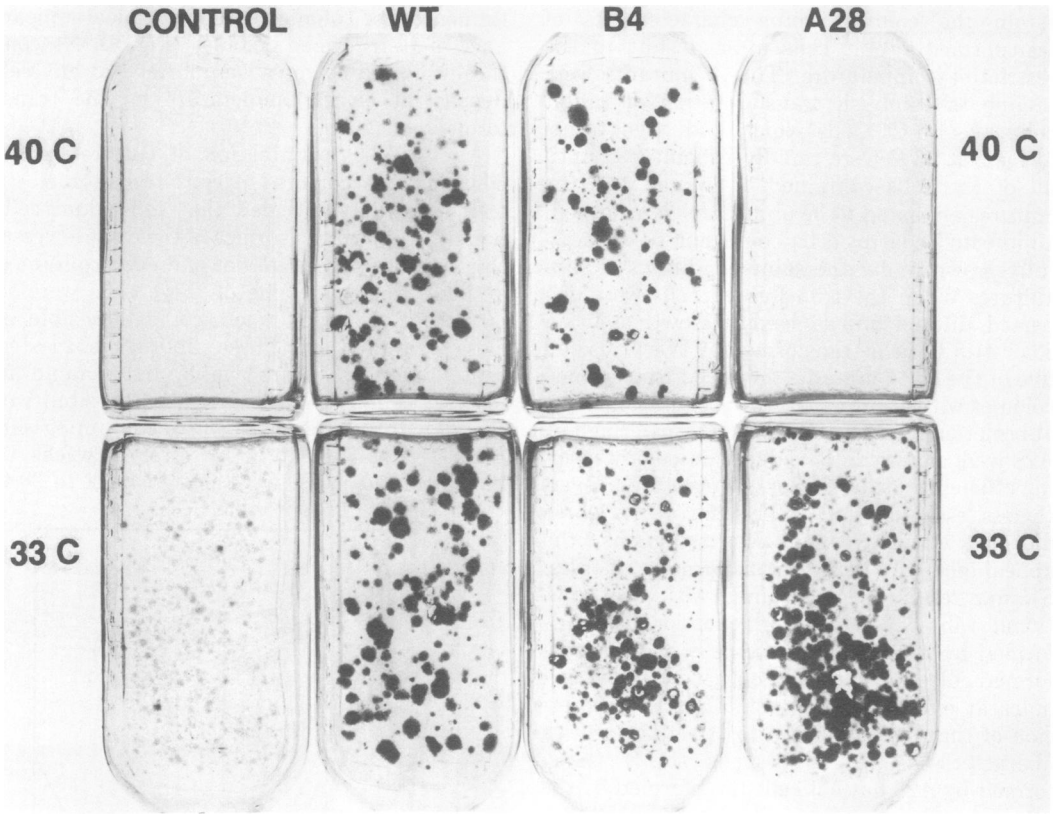

FIG. 2. *Appearance of colonies of nontransformed and transformed hamster embryo cells at 33 and 40 C. The samples are from the same experiment shown and described in Fig. 1. Uninfected cells and cells stably transformed by WT or mutant virus at 33 C were dispersed and cultured at the same low density at 33 or 40 C. The transforming virus and temperature of incubation are indicated in the figure.*

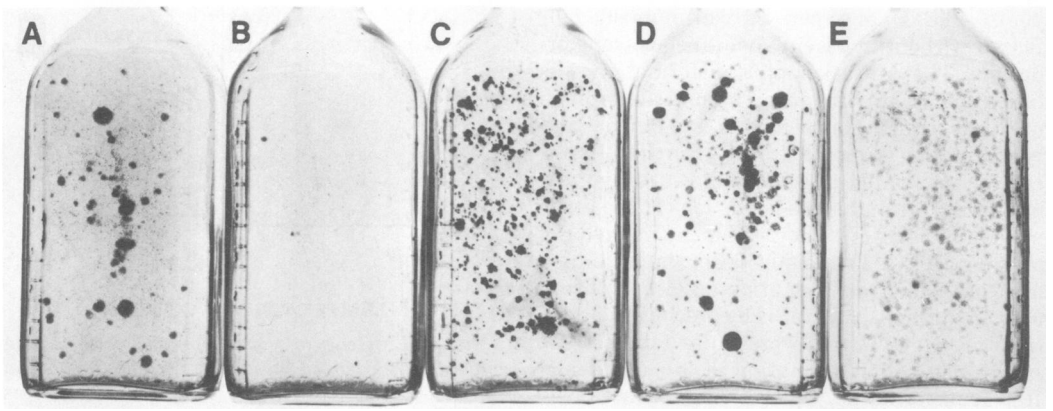

FIG. 3. *Reversibility of the temperature-sensitive inhibition of growth of hamster cells after stable transformation by A58 at 33 C. The samples are from the same experiment shown and described in Fig. 1 and 2. (A) A 10^{-2} dilution of A58 cells incubated 2 weeks at 40 C; (B) a 10^{-3} dilution of A58 cells incubated 2 weeks at 40 C; (C) a 10^{-3} dilution of A58 cells incubated 2 weeks at 40 C and then 2 weeks at 33 C; (D) a 10^{-5} dilution of A58 cells incubated 3 weeks at 33 C; and (E) a 10^{-4} dilution of nontransformed hamster cells incubated 3 weeks at 33 C. The microscopic appearance of selected colonies is shown in Fig. 4.*

and incubated for 2 weeks at that temperature. A small but significant proportion of the cells formed transformed colonies similar in appearance to those formed during continuous incubation at 33 C (Fig. 1 and 3).

Synthesis of T antigen and infectious virus by transformed cells. Each of the 3T3, rabbit, and hamster cell lines which had been stably transformed at 33 C was examined for T antigen synthesis and virus production at 33 C and at the appropriate restrictive temperature 1 week after a shift of confluent cultures to the higher temperature. In each transformed cell line at either temperature, essentially all of the cells contained T antigen as determined by fluorescent antibody assay. Transformed 3T3 cells and hamster embryo cells were free of infectious virus at both the permissive and restrictive temperatures. Transformed rabbit kidney cells regularly produced small quantities (10^2 to 10^4 PFU/ml) of infectious virus at 33 C. Rabbit kidney cells transformed by WT virus, but not by the mutants, also produced similar quantities of virus at 41.5 C. Thus the fate of the transformed cells at the restrictive temperature did not seem to correlate in any way with either the synthesis of T antigen or the production of infectious virus.

DISCUSSION

The *A* function of SV40 is not only required to initiate the stable transformation of cells but may also have a significant effect on the capacity of transformed cells to form colonies after the establishment of stable transformation. Even though some populations of cells transformed by *A* mutants have a low efficiency of colony formation at low cell concentrations at the restrictive temperature, essentially all of the colonies which do form at higher cell concentrations have the appearance of dense transformed colonies. These findings could be explained in quite different ways. At one extreme of interpretation, the data could indicate that the mechanism by which the loss of *A* function reduces colony-forming efficiency of transformed cells is

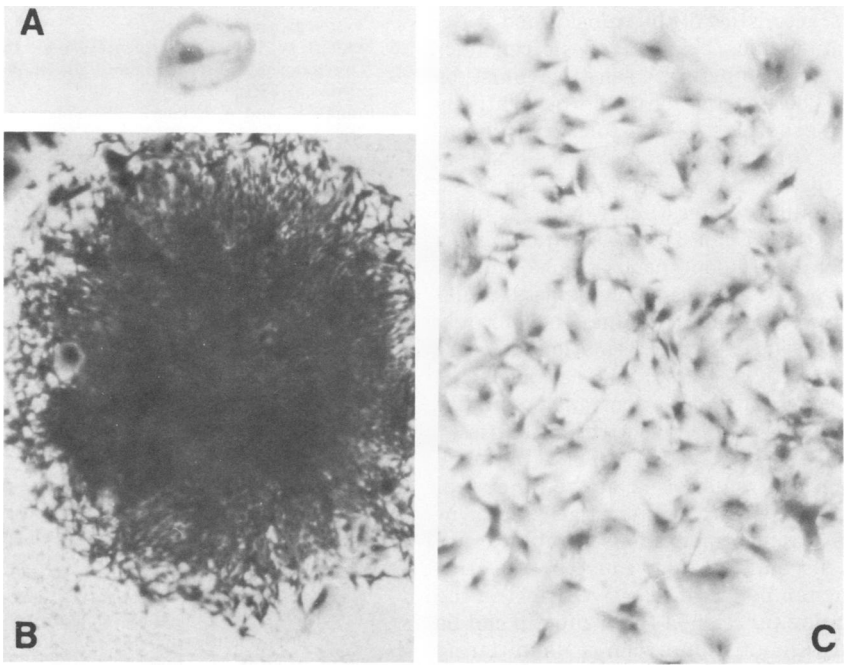

FIG. 4. *Photomicrographs showing the behavior of hamster embryo cells after the establishment of stable transformation by A58 at 33 C. The samples are from the same experiment shown in Fig. 1 to 3. Each sample was photographed at the same magnification. (A) A hamster embryo cell stably transformed by A58 at 33 C and then incubated in a dilute suspension of single cells for 2 weeks at 41 C without cell division; (B) formation of a transformed colony by a hamster embryo cell stably transformed by A58 at 33 C, incubated in a dilute suspension of single cells for 2 weeks at 41 C, and then incubated for 2 weeks at 33 C to show the reversibility of the temperature-dependent inhibition of growth; and (C) appearance of a nontransformed colony of hamster embryo cells after 3 weeks at 33 C.*

different from the mechanism by which SV40 induces the transformed state. At the other extreme of interpretation, the findings could indicate that the capacity to form colonies under adverse circumstances reflects the most critical step in the transformation process. In this case, the complete phenotype of transformation would be expressed by any cells capable of forming colonies.

The evidence that the temperature-sensitive *A* function is responsible for the temperature-sensitive growth of the transformed cells will remain circumstantial until cells of an isogenic clone transformed by either the *A* mutants or WT virus are compared. It seems unlikely, however, that the *A* mutants and WT virus are selectively transforming different variants in the same cell population. Furthermore, the temperature-sensitive changes of transformed Chinese hamster, rat embryo, and human cells, as well as Syrian hamster cells, reported in the accompanying studies (2, 10, 11) indicate that the altered behavior of cells transformed by *A* mutants is not an isolated phenomenon.

If the *A* function is required to retain certain growth characteristics of the transformed state, then the anomalous behavior of the cell lines which are not temperature-sensitive must be explained. The explanation could be relatively trivial. For example, spontaneous transformation may be superimposed on some cells subsequent to the virus-induced growth changes. Such a secondary alteration would be quite plausible in the case of 3T3 cells, which have a significant rate of spontaneous transformation depending on conditions of cell passage and growth. However, rabbit kidney cells rarely undergo spontaneous transformation and are usually very difficult to establish as continuous lines. A second and more important possibility is that some host cells selectively "suppress" temperature-sensitive changes in viral proteins. This suppression would occur at the functional level inasmuch as early viral antigens are synthesized in transformed mouse, rabbit, and hamster cells and, further, must be selective because the temperature-sensitive defect in the colony-forming function of the *A* protein can be suppressed without suppression of its initiating function. Thus, the anomalous behavior of the *A* protein in different hosts may reflect a dual function of the *A* gene.

A satisfactory explanation for the findings reported here will require the identification, isolation, and characterization of the *A* protein and a study of its interaction with different host cells, as well as a better understanding of growth control in normal cells.

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LITERATURE CITED

1. Black, P. H. 1966. Transformation of mouse cell line 3T3 by SV40: dose response relationship and correlation with SV40 tumor antigen production. Virology **28**:760–763.
2. Brugge, J. S., and J. S. Butel. 1975. Role of simian virus 40 gene *A* function in the maintenance of transformation. J. Virol. **15**:619–635.
3. Chou, J. Y., J. Avila, and R. G. Martin. 1974. Viral DNA synthesis in cells infected by temperature-sensitive mutants of simian virus 40. J. Virol. **14**:116–124.
4. Cowan, K., P. Tegtmeyer, and D. D. Anthony. 1973. Relationship of replication and transcription of simian virus 40. Proc. Nat. Acad. Sci. U.S.A. **70**:1927–1930.
5. DiMayorca, G., J. Callender, G. Marin, and R. Giordano. 1969. Temperature-sensitive mutants of polyoma virus. Virology **38**:126–133.
6. Eckhart, W. 1969. Complementation and transformation by temperature-sensitive mutants of polyoma virus. Virology **38**:120–125.
7. Franke, B., and W. Eckhart. 1973. Polyoma gene function required for viral DNA synthesis. Virology **55**:127–135.
8. Fried, M. 1965. Cell transforming ability of a temperature-sensitive mutant of polyoma virus. Proc. Nat. Acad. Sci. U.S.A. **53**:486–491.
9. Kimura, G., and R. Dulbecco. 1972. A temperature-sensitive mutant of simian virus 40 affecting transforming ability. Virology **52**:529–534.
10. 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.
11. Osborne, M., and K. Weber. 1975. Simian virus 40 gene *A* function and maintenance of transformation. J. Virol. **15**:636–644.
12. Pope, J. H., and W. P. Rowe. 1964. Detection of specific antigen in SV40 transformed cells by immunofluorescence. J. Exp. Med. **120**:121–128.
13. Robb, J. A., and K. Huebner. 1973. Effect of cell chromosome number on simian virus 40 replication. Exp. Cell Res. **81**:120–126.
14. Tegtmeyer, P. 1972. Simian virus 40 deoxyribonucleic acid synthesis: the viral replicon. J. Virol. **10**:591–598.
15. Tegtmeyer, P., C. Dohan, and C. Reznikoff. 1970. Inactivating and mutagenic effects of nitrosoguanidine on simian virus 40. Proc. Nat. Acad. Sci. U.S.A. **66**:745–752.
16. Tegtmeyer, P., and H. L. Ozer. 1971. Temperature-sensitive mutants of simian virus 40: infection of permissive cells. J. Virol. **8**:516–524.
17. Todaro, G. J., and H. Green. 1966. High frequency of SV40 transformation of mouse cell line 3T3. Virology **28**:756–759.