JOURNAL OF VIROLOGY, Mar. 1978, p. 860-870
0022-538X/78/0025-0860$02.00/0

Vol. 25, No. 3
Printed in U.S.A.

Transformation of BALB/c-3T3 Cells by *tsA* Mutants of Simian Virus 40: Temperature Sensitivity of the Transformed Phenotype and Retransformation by Wild-Type Virus

WILLIAM W. BROCKMAN

Department of Microbiology, University of Michigan Medical School, Ann Arbor, Michigan 48109

Received for publication 11 October 1977

The function of the *A* gene of simian virus 40 (SV40) in transformation of BALB/c-3T3 cells was investigated by infecting at the permissive temperature with wild-type SV40 and with six *tsA* mutants whose mutation sites map at different positions in the early region of the SV40 genome. Cloned transformants were then characterized as to the temperature sensitivity of the transformed phenotype. Of 16 *tsA* transformants, 15 were temperature sensitive for the ability to overgrow a monolayer of normal cells, whereas three of three wild-type transformants were not. This pattern of temperature sensitivity of the transformed phenotype was also observed when selected clones were assessed for the ability to grow in soft agar and in medium containing low concentration of serum. The temperature resistance of the one exceptional *tsA* transformant could be attributed neither to the location of the mutation site in the transforming virus nor to transformation by a revertant virus. This temperature-resistant *tsA* transformant, however, was demonstrated to contain a higher intracellular concentration of SV40 T antigen than a temperature-sensitive line transformed by the same *tsA* mutant. A *tsA* transformant displaying the untransformed phenotype at the nonpermissive temperature was found to be susceptible to retransformation by wild-type virus at this temperature, demonstrating that the temperature sensitivity of the *tsA* transformants is due to the viral mutation and not to a cellular defect. These results indicate that continuous expression of the product of the SV40 *A* gene is required to maintain the transformed phenotype in BALB/c-3T3 cells.

Expression of the early region of the genome of simian virus 40 (SV40) has been shown to be both necessary (21, 39) and sufficient (1, 37) for transformation of nonpermissive and semipermissive cells. Recently, several laboratories (7, 22, 28, 31, 40) examined the temperature sensitivity of the transformed phenotype of cells transformed by early temperature-sensitive (*tsA*) mutants of SV40 in an attempt to define the role of SV40 in the maintenance of the transformed state. A major discrepancy within these studies was the difference observed by two laboratories in regard to the temperature sensitivity of *tsA*-transformed mouse cells (7, 40). Brugge and Butel (7) found *tsA* transformants to be temperature sensitive; Tegtmeyer (40), however, did not find this to be the case. Since SV40-infected mouse cells have served as the basis for much of our understanding of the interaction between SV40 and nonpermissive cells, it is crucial to resolve this discrepancy. Although several differences in experimental procedure might account for the different observations reported by the two laboratories, one striking difference is that the cells studied by Brugge and Butel (7) were transformed by *tsA7*, whereas those studied by Tegtmeyer (40) were transformed by *tsA28* and *A30*. These three *tsA* mutants map at different positions in the early region of the SV40 genome (24, 26; Fig. 1), suggesting the possibility that the reported differences in temperature sensitivity of the transformed phenotype result from differences in the site of the defect in the transforming virus.

This communication reports the result of analysis of 19 clones of BALB/c-3T3 mouse cells, each individually transformed by wild-type (wt) SV40 or by one of six *tsA* mutants whose mutation sites map at different positions within the early region of the SV40 genome. The majority of the *tsA* transformants, but none of the wt transformants, are temperature sensitive for maintenance of transformation. Differences in the location of the defect in *tsA* mutants do not appear to be responsible for differences in the temperature sensitivity of the transformed phenotype. On the other hand, analysis of two lines transformed by the same *tsA* mutant suggests

that the degree of temperature sensitivity may depend on the amount of SV40 T antigen present. Finally, temperature-sensitive *tsA* transformants that have been superinfected with wt SV40 are resistant to temperature inhibition of the transformed state, demonstrating that the *ts* defect in the original *tsA* transformants lies in a viral rather than a cellular gene. The results reported here indicate that the continued expression of the *A* gene function is necessary for the maintenance of SV40-mediated transformation of BALB/c-3T3 cells.

MATERIALS AND METHODS

Virus and cells. wt SV40 strain 776 was derived from plaque-purified virus as described (13). wt strain VA45-54 and temperature-sensitive mutants of SV40 (*tsA7*, *A30*, *A209*, *A241*, *A255*, and *A276*) were generously supplied by Peter Tegtmeyer (39, 41) or by Robert Martin and Janice Chou (10). Stocks of the *ts* mutants were prepared at 33°C by inoculating confluent monolayers of the BSC-1 line of African green monkey cells at multiplicities of infection (MOI) between 0.002 and 0.01 plaque-forming units (PFU) per cell. BSC-40 cells (6) were used for titrating virus at 33 and 39.5°C. BALB/c-3T3 cells, obtained from Daniel Nathans, were grown in minimal Eagle medium (MEM) with 20% fetal calf serum (FCS).

Cell transformation. Subconfluent BALB/c-3T3 cells (10^5 cells) growing in 1.5-cm wells at 33°C were infected at a series of multiplicities with wt SV40 (strain 776) or one of the *ts* mutants. Twenty-four hours later, the cells were trypsinized and transferred to a 10-cm petri dish in MEM with 5% FCS. After 4 to 6 weeks, colonies overgrowing the monolayers were scored as transformants. Several colonies from each virus infection were isolated and cloned at 33°C by plating a dilute cell suspension in MEM with 10% FCS and selecting colonies originating from single cells.

Detection of SV40 T antigen. Indirect immunofluorescence was performed with anti-T serum and fluorescein-tagged anti-hamster serum generously provided by the Office of Program Resources and Logistics, National Cancer Institute. Complement fixation (CF) assays for the quantitation of T antigen in cell extracts were performed as described by Anderson et al. (3). Detection and quantitation of T antigen was also performed by the protein A-immune complex assay as described by Crawford and Lane (12). Briefly, anti-T serum was added in excess to cell extracts, the resulting immune complex was isolated on glass fiber filters, and the amount of complexed immunoglobulin assessed by addition of ^{125}I-labeled protein A (a gift from J. L. Claflin) from *Staphylococcus aureus*. The amount of protein A bound is related to the amount of immunoglobulin and hence of antigen on the filter. Protein A binding is expressed as the percent of ^{125}I counts added to the immune complex-containing filter which remains bound to the filter after extensive washing.

Colony formation on monolayers of normal cells. Cells growing at 33°C were trypsinized and plated at several concentrations (between 40 and 2 × 10^4 cells per well) onto confluent monolayers of BALB/c-3T3 cells in 1.5-cm wells. Duplicate cultures were incubated at 33 or 39.3°C, and the media were changed every third day. Overgrowth of the monolayer was monitored by examination under a dissecting microscope, and, on the tenth day, cultures were stained with Evan blue as described by Martin and Chou (28).

Colony formation in soft agar. The technique for colony formation in soft agar was based on that of MacPherson and Montagnier (27). Basal layers (0.4 ml) of 0.75% Difco agar in MEM with 12.5% FCS were formed in 1.6-cm wells, followed by the addition of 0.3 ml of 0.3% agar in MEM with 12.5% FCS containing various dilutions of cells (between 50 and 5 × 10^3 cells) which had been growing at 33°C. Duplicate cultures were incubated at 33° or 39.3°C, and colony counts were performed after 2 weeks by using a dissecting microscope. Only colonies containing more than 10 cells were scored.

Serum requirement for cell growth. Approximately 10^4 cells that had been growing at 33°C were seeded into 1.6-cm wells and allowed to attach at 33°C in MEM with 10% FCS. Twelve hours later, medium was replaced with MEM containing 1, 2, 5, 10, or 20% FCS, and replicate cultures were incubated at 33 or 39.3°C. Cell counts and medium changes were performed at daily intervals.

Cell division and incorporation of thymidine into DNA after shift from nonpermissive to permissive temperature. Cells that had been growing at 33°C were seeded in replicate 1.6-cm wells (10^4 cells per well) in MEM with 1% FCS and incubated at 33° and 39.5°C. After 44 h, medium was replaced with 1 ml of MEM containing 1% FCS per well and 1 μCi of [^{3}H]thymidine (0.2 Ci/mmol). Nine hours after addition of the [^{3}H]thymidine, one-half of the cultures at 39.5°C were shifted to 33°C. Over the next 3 days, cell counts and measurements of [^{3}H]thymidine incorporation were made on replicate cultures at approximately 12-h intervals. To assess [^{3}H]thymidine incorporation into acid-precipitable material, monolayers were rinsed with cold phosphate-buffered saline and lysed with 0.2 ml of 10 mM EDTA–0.6% sodium dodecyl sulfate. Lysates were transferred to Whatman GF/B filters which had been pretreated with 5% trichloroacetic acid. Filters were washed with cold 5% trichloroacetic acid followed by ethanol. Filters from duplicate samples were dried, counted, and averaged.

Rescue of SV40 from transformed cells. The procedure used to rescue SV40 from transformed cells was a modification of that previously described (37). Transformed 3T3 cells were seeded in 25-cm^2 flasks (10^5 cells per culture). After these cells had attached (6 h, 33°C), 3 × 10^6 BSC-40 cells were added to each flask, and the culture was incubated for 18 h at 33°C. The monolayer was then washed twice with cold MEM without serum and exposed to UV-inactivated Sendai virus (2,000 hemagglutinating units in 1 ml of MEM without serum) and incubated on ice for 20 min. At the end of the incubation, the Sendai virus was removed and the monolayer again was rinsed twice with cold MEM. MEM (2.5 ml) which had been pre-equilibrated at 33°C was added, and the culture was further incubated at 33°C for 60 min, after which 2.5 ml of MEM with 20% FCS was added. Twelve hours later,

the medium was replaced with MEM containing 2% FCS, and incubation continued at 33°C for 8 days. At this time, the cultures were frozen and thawed twice, and the resulting lysates were sonically disrupted in a Raytheon well sonicator for 4 min at maximum energy.

Endonuclease R analysis of DNA. Viral DNA was labeled with ^{32}P and then purified by Hirt extraction (18) and electrophoresis in 1.4% agarose gel slabs, as described elsewhere (6). Viral DNA recovered from agarose gels was concentrated by isopropyl alcohol precipitation, dissolved in 10 mM Tris-1 mM EDTA (pH 7.6), and subjected to digestion with endonuclease R *Hin*dII + III (14). *Hin* fragments were separated by electrophoresis in 4% polyacrylamide gel slabs, and fragments were visualized by autoradiography.

RESULTS

***tsA* mutants used to induce transformation.** It is possible, as mentioned above, that *tsA* mutants that differ in the location of their defects might differ in the temperature sensitivity of the ability to maintain transformation. To examine this possibility, BALB/c-3T3 cells were transformed under permissive conditions by wt SV40 and by six *tsA* mutants whose mutation sites map at different positions in the early region. Cloned transformants were then characterized as to the temperature sensitivity of the transformed phenotype. The six *tsA* mutants used were *A*7, *A*30, *A*209, *A*241, *A*255, and *A*276 (10, 39, 41). A map of these mutants in relation to the *Hin*dII + III cleavage map of SV40 as determined by the marker rescue studies of Lai and Nathans (24, 26) is shown in Fig. 1. The mutation sites of *tsA*7 and *A*30 lie in *Hin* fragments B and H, respectively. The other four mutants, while all mapping within fragment I, have their mutation sites at different locations within this fragment, as determined by Lai and Nathans (26) using a *ts*/*ts* heteroduplex infectivity assay.

Transformation of BALB/c-3T3 cells. BALB/c-3T3 cells were infected with wt SV40 or with one of the six *tsA* mutants at various MOIs ranging from 0.25 to 500 (see Table 1). Cells infected with wt SV40, as well as those infected with the *ts* mutants, were maintained at 33°C, an essential condition if the temperature sensitivities of maintenance of wt- and *tsA*-induced transformation are to be compared. Individual colonies of transformed cells were detected some 4 to 6 weeks after infection as areas of dense overgrowth on uniformly confluent monolayers. Two to three colonies transformed by each virus were isolated, shown by indirect immunofluorescence assay to contain SV40 T antigen, and subcloned at 33°C. The various transformed clones used for further studies are listed in Table 1. The designation of each clone indicates the strain of SV40 used for transformation. Also shown are the virus-to-cell ratios (MOI) at which the original infections were performed. wt and all *ts* viruses transformed with approximately the same frequency (about 10^5 PFU per transforming unit).

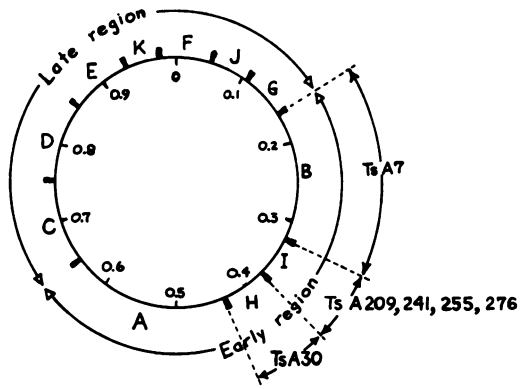

FIG. 1. *HindII + III cleavage map of SV40 genome (14) showing regions of early and late transcription (20) and regions in which the six tsA mutants used in this study have been mapped by marker rescue analysis (24, 26).*

TABLE 1. *Origin of transformed clones*

Transforming virus	MOI	Clone
wt	200	WTB1a
	200	WTB3b
	20	WTB4a
*tsA*7	500	*A*7B1a
	500	*A*7B4b
	5	*A*7B5b
*tsA*30	200	*A*30B3a
	200	*A*30B4b
*tsA*209	25	*A*209B1b
	25	*A*209B2b
	0.25	*A*209B4a
*tsA*241	200	*A*241B1c
	200	*A*241B2c
*tsA*255	250	*A*255B1b
	250	*A*255B2a
	25	*A*255B4a
*tsA*276	200	*A*276B2a
	200	*A*276B3a
	20	*A*276B4a

Temperature sensitivity of the ability to grow on monolayers and in soft agar. To assess the role of the SV40 *A* gene product in the maintenance of the transformed state in BALB/c-3T3 cells, the 19 transformants (Table 1) which had been transformed at 33°C (the permissive temperature for the *tsA* mutants)

were tested for temperature sensitivity of the transformed phenotype. This was done by plating the transformed cells on confluent monolayers of normal 3T3 cells and comparing their ability to form colonies at permissive and nonpermissive temperatures. Untransformed BALB/c-3T3 cells plated on plastic form colonies a single cell thick; when seeded onto a confluent monolayer, they are unable to form colonies. Transformed cells, on the other hand, have the ability to overgrow one another and can form dense colonies when seeded onto a normal monolayer. Using monolayer overgrowth as the primary criterion for the temperature sensitivity of the transformed phenotype has two advantages. First, it is a criterion similar to that used for the original selection of the transformed lines. Second, a nonselective analysis of SV40 transformation of mouse 3T3 cells by Risser and Pollack (35) has shown that SV40-infected 3T3 cells which display this property also possess the several other properties characteristic of standard SV40-transformed cells.

The results of one series of monolayer overgrowth experiments are shown in the first three columns of Table 2. In this and other studies reported in this communication, 33°C was taken as the permissive temperature and, unless otherwise noted, 39.3°C was taken as the restrictive temperature. Other experiments performed under similar conditions yielded similar results. Comparison of the efficiency of plating, or colony-forming ability, on monolayers at the permissive temperature (P) with that at the nonpermissive temperature (NP) for the 19 transformed lines reveals that the P/NP ratios were close to unity for the wt transformants, but 10^2 or greater for the majority of the *tsA* transformants. The three wt transformants, then, were temperature resistant for the ability to overgrow a monolayer, whereas most of the *tsA* transformants were temperature sensitive for this property. The striking exceptions were lines *A*7B1a and *A*30B3a, which were nearly as temperature resistant as the wt transformants.

As a further test of the temperature sensitivity of the transformed phenotype, anchorage dependence of multiplication of several of the cloned lines was tested at the permissive and nonpermissive temperatures. Untransformed BALB/c-3T3 cells divide only if they are attached to a solid surface. Transformed cells, on the other hand, can grow when suspended in an agar gel. As seen in the last three columns of Table 2, the wt transformant was able to grow in soft agar nearly as well at the permissive as at the nonpermissive temperature (P/NP = 1.8). In contrast, the P/NP ratio for five of the *tsA*

TABLE 2. *Effect of temperature on ability of transformed cells to grow on normal monolayers and in soft agar*

Cell line	EOP[a] on monolayer						EOP[a] in soft agar		
	Expt 1			Expt 2					
	P	NP[b]	P/NP	P	NP[c]	P/NP	P	NP[b]	P/NP
WTB1a	11.3	13.8	0.82				29.0	15.7	18
WTB3b	35.0	30.0	1.17	36.7	30.0	1.22			
WTB4a	22.5	30.0	0.75						
*A*7B1a	10.0	3.0	3.33	18.4	0.05	368	50.0	7.2	6.9
*A*7B4b	40.7	0.38	107	33.3	0.33	100	31.3	0.30	104
*A*7B5b	7.5	≤0.01	≥750						
*A*30B3a	42.5	17.5	2.43	25.0	8.4	2.97	68.0	3.8	17.9
*A*30B4b	2.5	0.01	250	3.3	≤0.01	330	43.9	0.60	73.2
*A*209B1b	2.5	≤0.01	≥250						
*A*209B2b	2.0	≤0.01	≥200						
*A*209B4a	12.5	0.03	417				27.5	0.25	110
*A*241B1c	47.5	0.13	365						
*A*241B2c	20.0	0.03	667						
*A*255B1b	45.0	0.15	300				29.8	0.12	248
*A*255B2a	5.6	0.06	93						
*A*255B4a	8.8	0.03	293				12.2	0.03	407
*A*276B2a	25.0	0.05	500						
*A*276B3a	1.5	≤0.01	≥150						
*A*276B4a	10.0	≤0.01	≥1,000						
BALB/c-3T3	<0.01	≤0.01					≤0.001	≤0.001	

[a] EOP, Efficiency of plating, expressed as number of colonies per 100 cells plated.
[b] 39.3°C used as the nonpermissive temperature
[c] 39.8°C used as the nonpermissive temperature.

transformants tested ranged from 73 to 406. Lines *A*7B1a and *A*30B3a, however, again were characterized by relatively low P/NP ratios (6.9 and 17.9, respectively).

Since *tsA*7 is known to be a rather leaky mutant (39; Table 3), the ability of the relatively temperature-resistant *tsA*7 transformant (*A*7B1a) to overgrow a monolayer was assessed again using a higher temperature for the restrictive condition. The center three columns of Table 2 show the results of a set of monolayer overgrowth assays in which 39.8°C was used as the nonpermissive temperature. Under this condition, *A*7B1a was temperature sensitive. The difference in temperature sensitivity of *A*7B1a and the two other *A*7 lines, therefore, may reflect differences in the intracellular concentration of a relatively leaky *ts* protein. Line *A*30B3a, however, was still temperature resistant at the elevated nonpermissive temperature. Thus, three of three BALB/c-3T3 lines independently transformed by wt SV40 were temperature resistant for the transformed phenotype as assayed here, whereas 15 of 16 *tsA* transformants were temperature sensitive. The temperature resistance of the one *tsA* transformant, *A*30B3a, cannot be attributed to a difference in the mutation site in the transforming virus, since another *tsA*30 transformant, *A*30B4b, was temperature sensitive. Possible explanations for the temperature resistance of *A*30B3a are examined below.

Temperature sensitivity of the ability to grow in low concentrations of serum. Untransformed cells require higher concentration of serum for cell division than do transformed cells. To determine whether continued expression of the SV40 gene *A* function is required for the ability of transformed BALB/c-3T3 cells to grow in low serum, several of the transformed lines were plated in medium containing various concentrations of serum and were monitored for growth at the permissive and nonpermissive temperatures. At both temperatures, the rate of growth of untransformed, wt-transformed, and *tsA*-transformed cells decreased with decreasing serum concentration. The growth rate of the wt transformants at any given serum concentration and temperature combination was greater than that of normal cells. At the permissive temperature, growth of the *tsA* transformants resembled that of the wt transformants, whereas, at the nonpermissive temperature, growth of the *tsA* transformants approached that of untransformed cells. The patterns of growth in medium containing 1% FCS are shown in Fig. 2. At this serum concentration, little growth of untransformed BALB/c-3T3 cells was observed at either temperature. The wt transformant grew at both temperatures, dividing more rapidly at the elevated temperature. Growth of the two *tsA* transformants tested, however, was markedly reduced at the nonpermissive temperature. Reduced serum requirement for growth, then, is another parameter of transformation that is temperature sensitive in *tsA*-transformed cells.

Reversibility of temperature inhibition

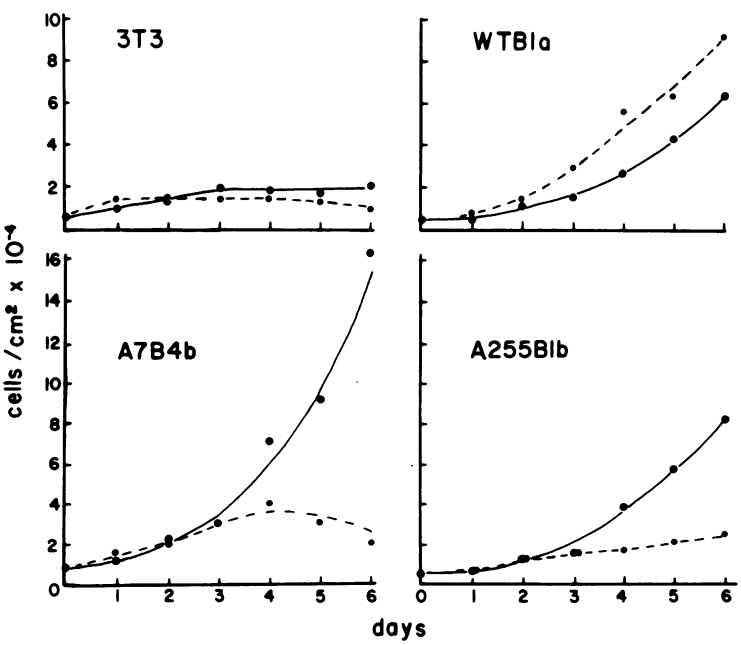

FIG. 2. *Growth of normal and transformed BALB/c-3T3 cells in medium containing 1% FCS.* (O——O) *growth at 33°C;* (●----●) *growth at 39.3°C.*

of cell growth and DNA synthesis. To determine whether temperature inhibition of the transformed phenotype of *tsA* transformants is reversible, *tsA*-transformed cells whose growth in 1% FCS had been inhibited by incubation at 39.5°C were shifted to 33°C and monitored for both cell division and incorporation of [^{3}H]thymidine into acid-precipitable material (Fig. 3). As anticipated from the results presented in the preceding section, little thymidine incorporation or cell division occurred in untransformed BALB/c-3T3 cells at either temperature or in the *tsA* transformant at the elevated temperature. DNA synthesis and cell division proceeded at both temperatures in the wt-transformed line, but only at the permissive temperature in the *tsA* transformant. When the *tsA* transformant incubated at the nonpermissive temperature was shifted to the permissive temperature, there was a resumption of thymidine incorporation and cell division. The transformed phenotype, then, as defined in this case by the ability to incorporate thymidine and undergo cell division in low serum, is reversibly inhibited at the nonpermissive temperature in *tsA*-transformed 3T3 cells. It is also of interest that on temperature shift the *tsA*-transformed cells appeared to resume both DNA synthesis and cell division in a nonrandom fashion (see Fig. 3). This suggests that the majority of these cells at the nonpermissive temperature were blocked at the same point in the cell cycle.

Rescue of virus from transformed cells. As noted above, the transformed phenotype of one *tsA* transformant, *A*30B3a, unlike those of the 15 other *tsA* transformants, continues to be expressed at the nonpermissive temperature. This difference cannot be explained by differences in the mutation sites in the transforming viruses. Alternatively, a possible explanation for the temperature resistance of line *A*30B3a is that it was transformed by a revertant of *tsA*30. To investigate this possibility, virus was rescued at 33°C from this line by Sendai virus-mediated fusion with permissive cells (BSC-40) and titrated at 33° and 39.5°C. Table 3 shows the titer of virus rescued from this and four other lines when assayed at the two temperatures. Virus rescued from the temperature-resistant cell line *A*30B3a was temperature sensitive for growth, as was the virus rescued from the other *tsA*30 transformant and the two *tsA*7 transformants. While this result suggests that the temperature resistance of *A*30B3a is not attributable to transformation by a revertant of *tsA*30, the possibility remains that a revertant genome, as well as the *ts* genome, is present in this line (*A*30B3a was transformed at an MOI of 200 PFU per cell) and that only the latter was recovered in the rescue experiment. This possibility has been investigated as described in the following section.

Thermolability and concentration of T

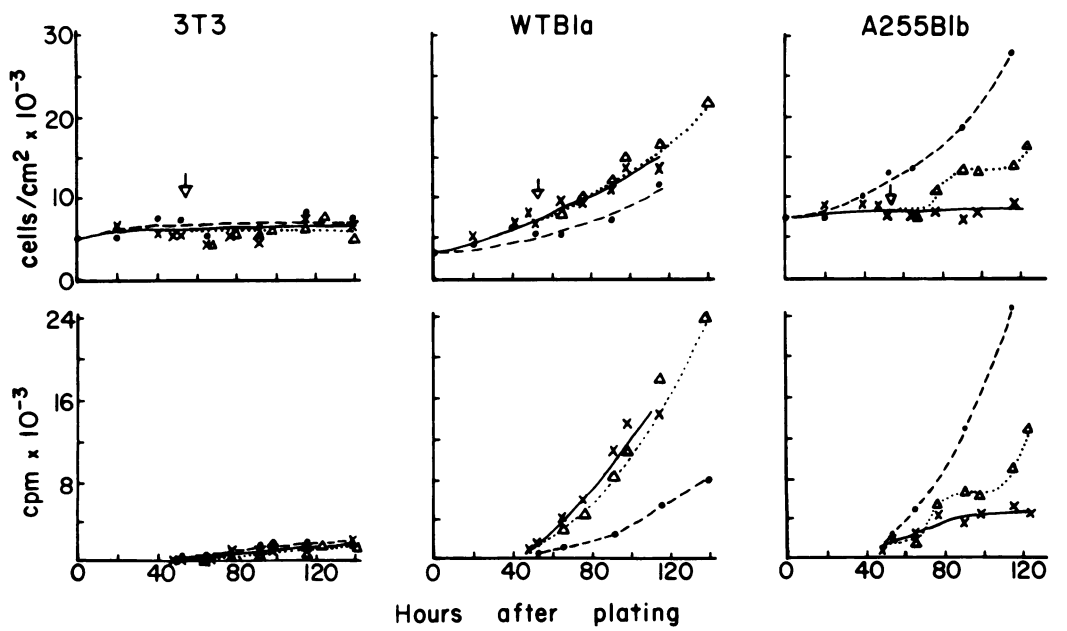

FIG. 3. *Cell division and incorporation of [^{3}H]thymidine into DNA following shift from nonpermissive to permissive temperature. [^{3}H]thymidine was added to cultures grown at 33 (●- - -●) and 39.5°C (×——×) at 44 h after plating. One-half of the cultures grown at 39.5°C were shifted (↓) to 33°C (△· · · · · △) at 53 h after plating. Each point represents the average value of duplicate measurements.*

TABLE 3. *Temperature sensitivity of virus rescued from transformed lines*

Line	PFU/ml[a]		
	33°C	39.5°C	33/39.5°C
WTB3b	7.0×10^5	9.0×10^5	0.8
*A*7B1a	1.5×10^6	2.0×10^2	8.0×10^3
*A*7B4b	2.6×10^6	1.0×10^3	2.6×10^3
*A*30B3a	3.0×10^5	<2	$>1.5 \times 10^5$
*A*30B4b	1.0×10^5	<2	$>5.0 \times 10^4$

[a] Plaques scored 11 days postinfection at 39.5°C and 18 days postinfection at 33°C.

antigen in *tsA* transformants. Evidence that SV40 T antigen is the *A* gene product has been derived from analysis of in vitro translation products of SV40 RNA (32), from characterization of T antigen induced by an SV40 deletion mutant (36), and from analysis of alterations in the behavior of T antigen in *tsA* infection (23, 31, 42, 43). SV40 T antigen extracted from permissive cells infected with *tsA* mutants and assayed by CF has been shown to be more thermolabile than T antigen extracted from permissive cells during wt infection (2, 23). This thermolability can also be demonstrated in T antigen extracted from *tsA*-transformed BALB/c-3T3 cells, as shown in Fig. 4. T antigen extracted from cells grown at 33°C was incubated for various times at 39.5°C and then assayed for complement-fixing activity. T antigen extracted from both the temperature-sensitive line *A*30B4b and the temperature-resistant line *A*30B3a showed similar kinetics of inactivation (Fig. 4), both being approximately six times more labile than T antigen extracted from the two wt transformants. The heat lability of T antigen from line *A*30B3a indicates that a revertant SV40 genome is not expressed in this line.

T antigen from both lines *A*30B3a and *A*30B4b is heat labile. To determine whether the difference in the temperature sensitivity of the transformed phenotype in these two lines might be due to a difference in intracellular concentrations of the *A* gene product, T antigen levels were quantitated by the *Staphylococcus* protein A assay of T antigen-containing immune complexes (12). This assay proved to be as sensitive as, and more reproducible than, the CF assay for T antigen. Cellular levels of T antigen in *A*30B3a and *A*30B4b were quantitated by monitoring formation of immune complex when increasing amounts of cell extract were added to a fixed amount of anti-T serum (Fig. 5). With an increasing number of cells represented in the immunoprecipitation reaction, the percent of ^{125}I-labeled protein A bound increased, reaching the maximum of about 44% (Fig. 5). By determining the number of cells that give one-half maximum ^{125}I binding, it is possible to compare the T-antigen level per cell in lines *A*30B3a and *A*30B4b. In this assay, the cellular concentration of T antigen in *A*30B3a was 1.7 times that in *A*30B4b when the cells were grown at 33°C. This difference was amplified when the cells were grown at 39.3°C, the level in *A*30B3a then being

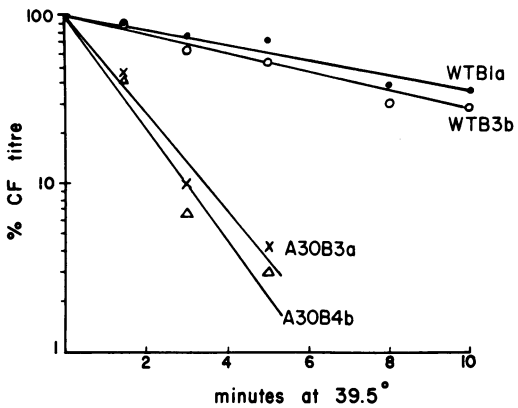

FIG. 4. *Heat inactivation of SV40 T antigen extracted from two wt and two tsA30 transformants. T antigen extracted from cells grown at 33°C was incubated at 39.5°C. At various time intervals, samples were cooled rapidly to 0°C and subsequently assayed by CF test (3). The results are plotted as a percentage of the CF titer at zero time of incubation.*

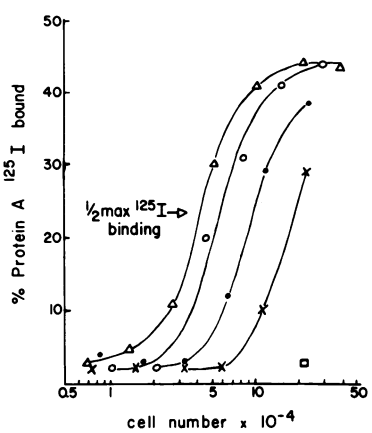

FIG. 5. *T-antigen levels in transformed cells as determined by the protein A-immune complex assay (12). 2 × 10^6 A30B3a and A30B4b cells, which had been growing for 3 days at 33 and 39.3°C, were plated in 10-cm dishes and grown to densities of 10^6 cells per dish at these same temperatures. Extracts from A30B3a (△) and A30B4b (×) cells grown at 39.3°C and from A30B3a (○), A30B4b (●), and untransformed BALB/c-3T3 (□) cells grown at 33°C were prepared, and serial dilutions were assayed for T antigen by protein A binding to T antigen-antibody complexes as described in the text. The results are plotted as a function of the number of cells represented in each extract dilution assayed.*

4.8 times that in A30B4b. While this difference varied from experiment to experiment, the temperature-resistant line, A30B3a, invariably was found to have a somewhat higher concentration of T antigen than the temperature-sensitive line, A30B4b, ranging from 1.5- to 3-fold at 33°C and from 3- to 6-fold at 39.3°C. These findings suggest the possibility that line A30B3a is temperature resistant for the transformed phenotype because it contains increased levels of T antigen, as a result of either increased production of or intracellular stabilization of the otherwise thermolabile T antigen. It is of interest that Tenen et al. (44) have reported that a *tsA*-transformed Chinese hamster lung cell line that is temperature resistant for the ability to overgrow a normal monolayer contains more T antigen by CF assay than other *tsA*-transformed Chinese hamster lung cells that are temperature sensitive for this property.

Retransformation of *tsA* transformants. It is unlikely that the various manifestations of the transformed phenotype are exclusively under the control of viral genes. Cellular defects might, therefore, result in temperature sensitivity of the transformed phenotype. Indeed, using a special selection procedure, Renger and Basilico (34) isolated SV40-transformed 3T3 cells that express the transformed phenotype in a temperature-sensitive manner apparently because they contain mutations in cellular genes. Thus, although the statistics of the present study (15 out of 16 *tsA* transformants were temperature sensitive for transformation; three of three wt transformants were not) strongly suggest that the temperature sensitivity of the *tsA* transformants is due to a viral defect, it would be desirable to demonstrate this in a more direct fashion. For this reason, I have sought to determine whether *tsA* transformants grown at the restrictive temperature (and now having the untransformed phenotype) can be retransformed by wt SV40. If retransformation can be demonstrated, then the *ts* defect in the original transformant must have been in a viral rather than a cellular gene.

The protocol used in the retransformation experiments is that described in Materials and Methods (cell transformation), except that *tsA*-transformed BALB/c-3T3 cells were infected at 39.5°C with wt virus. In one such retransformation experiment, a *tsA*-transformed line (A209B4a) was infected (and subsequently maintained) at the nonpermissive temperature with wt SV40 virus at an MOI of 100. Several colonies of piled-up cells were detected 18 days after infection, while no such colonies were observed on control dishes that were not infected with wt virus. Cells grown from one such colony were compared with A209B4a for their ability to form colonies on monolayers of normal 3T3 cells at 33° and 39.4°C. The ratio of efficiency of plating on monolayers at 33°C to that at 39.4°C was 0.86 for the reinfected cells, as compared with a ratio of ≥400 for A209B4a.

To determine whether these new temperature-resistant transformants were in fact derived from the original *tsA*209 transformants or, alternatively, from a subpopulation of cells which had lost the *tsA* genome, they were subcloned twice, after which their resident viral genomes were rescued by fusion with permissive cells and analyzed by restriction enzyme digestion. The restriction digest pattern of the DNA of the wt virus (VA45-54) used for the second infection differed slightly from that of the *tsA*209 virus (derived from wt strain 776) used for the original transformation (24). Because of this difference, restriction enzyme digestion of the rescued virus DNA could be utilized to determine whether both *tsA* and wt genomes were present in these new transformants. An electropherogram of *Hin*dII + III digests of the DNA from the virus rescued from the new temperature-resistant transformant is shown in Fig. 6. Also shown are digests of the genomes of the two wt strains, 776 and VA45-54. As described by Lai and Nathans (24), digestion of strain VA45-54 by *Hin*dII + III yields a C fragment that migrates more slowly, and an F fragment that migrates more rapidly, than the corresponding fragments from strain 776. Comparison of these two digest patterns with that of the viral DNA rescued from the new transformant reveals that the latter contains two C and two F fragments, corresponding to the C and F fragments of the two SV40 strains. Both *tsA*209 and VA45-54 genomes, then, are present in the new transformants, demonstrating that these cell lines were derived by superinfection of the original *tsA* transformant by wt virus. This is further evidence that the temperature sensitivity of BALB/c-3T3 *tsA* transformants is due to a viral defect and that the continued expression of the SV40 *A* gene function is required for maintenance of the transformed state.

DISCUSSION

Studies with *ts* mutants of SV40 initially demonstrated the essential role in transformation of the product of the early SV40 gene *A* (21, 39). In those studies, *tsA* mutants were found to be incapable of producing cellular transformation if infection was performed at high (nonpermissive) temperatures. More recently, several studies (7, 22, 28, 31, 40) have been performed in an attempt to determine whether continued expression of the *A* gene product is required to maintain the transformed state. In these latter studies, semi-

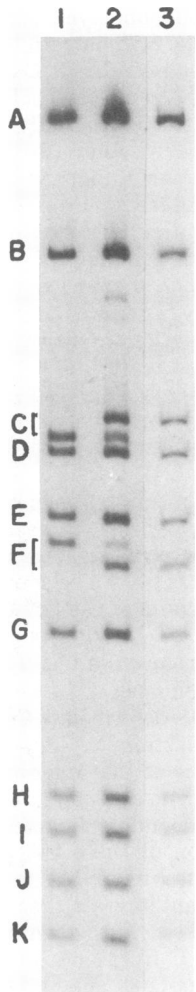

FIG. 6. *Hin digest patterns of wt 776 (column 1) and wt VA45-54 (column 3) DNA and of the DNA from virus rescued from wt-transformed A209B4a (column 2); autoradiogram of [^{32}P]DNA fragments following electrophoresis. Fragments corresponding to each wt fragment are labeled A through K. The unlabeled fragments (migrating between fragments B and C) from the rescued virus DNA probably are derived from minor defective DNA species generated during rescue.*

permissive and nonpermissive cells were transformed by *ts* mutants at the permissive low temperature and then assayed for various manifestations of the transformed phenotype at the permissive and nonpermissive temperatures. Under these conditions, *tsA*-transformed rat embryo cells (22, 31) and the majority of *tsA*-transformed hamster cell lines (7, 28, 40), but only a minority of *tsA*-transformed rabbit kidney cell lines (40), were found to be temperature sensitive for the transformation parameters tested. The two groups which examined *tsA*-transformed mouse cells observed different phenomena. Brugge and Butel (7) found the transformed phenotype reverted on shift to the nonpermissive temperature, whereas Tegtmeyer (40) did not detect such reversion. In the present study, 19 clones of SV40-transformed BALB/c-3T3 mouse cells have been analyzed for temperature sensitivity of the transformed phenotype. By utilizing the same criterion (i.e., ability to overgrow a monolayer of normal cells) as was used for the original selection of the transformed lines, 15 of 16 *tsA* transformants were temperature sensitive for maintenance of transformation, whereas three of three wt transformants were temperature resistant. This pattern of temperature sensitivity was confirmed when selected clones were assessed for ability to grow in soft agar and in medium containing low concentration of serum.

The results of these experiments imply that the protein encoded by the SV40 *A* gene is required to maintain the transformed phenotype in BALB/c-3T3 cells. This conclusion is strengthened by the results of the retransformation experiment. Since temperature-sensitive *tsA* transformants become temperature resistant on retransformation by wt virus, the temperature-sensitive defect must lie in the product of a viral and not a cellular gene. The retransformation phenomenon is also of interest as a potential means of analyzing mechanisms of integration of viral DNA into cellular chromosomes. If such integration can occur by homologous recombination, then it might be expected that the frequency of retransformation will be greater than that of the primary transformation event. The retransformation phenomenon also offers a means for analyzing the relationship between site of integration and transforming capacity. It will be of interest, therefore, to determine both the frequency of retransformation and the site of integration of the initial and retransforming virus genomes.

In addition to its role in transformation, the SV40 gene *A* product is required for the initiation of viral DNA synthesis (9, 39). This gene also appears to be required for SV40-induced stimulation of cellular DNA synthesis, although studies with *tsA* mutants show this function to be much less temperature sensitive than initiation of viral DNA replication (11, 38). Furthermore, studies with certain deletion mutants of SV40 (5, 25) have shown that the early region of the SV40 genome is both necessary and sufficient for performing all three of these functions (37). It is possible that these three functions are manifestations of a single activity of a viral gene product (e.g., binding of the *A* gene product to DNA [8, 19, 33]). Alternatively, the *A* gene pro-

tein may express more than one activity, or the early region may code for more than one independently acting polypeptide. The existence of separate functional segments of the early region of the SV40 genome, one responsible for DNA replication and another for transformation, might explain the differences reported by Tegtmeyer (40) and by Brugge and Butel (7) in regard to the requirement of continued *A* gene expression for the maintenance of transformation in mouse cells. *tsA*28 and *tsA*30, the mutants used by Tegtmeyer for transformation of mouse cells, map in *Hin* fragments I and H, respectively, of the SV40 genome, while *tsA*7, the mutant used by Brugge and Butel, is the only *tsA* mutant that maps in *Hin* fragment B (24; see Fig. 1). The region included within *Hin* fragments H, I, and B then might be required for viral DNA replication (the criterion used for designation of *tsA* mutants), while only a region within *Hin* fragment B need be expressed to maintain transformation in mouse cells. This possibility has been investigated in the experiments reported in this communication. BALB/c-3T3 cells were transformed by six *tsA* mutants which map at distinct positions within *Hin* fragments H, I, and B (see Fig. 1). Clones transformed by each of these *tsA* mutants were temperature sensitive for maintenance of transformation. One of the 16 *tsA*-transformed clones was temperature resistant. This temperature resistance, however, cannot be attributed to the location of the *ts* defect in the transforming virus. Although the cause of this temperature resistance is not known, data is presented which is consistent with the possibility that this exceptional case may be related to factors affecting the synthesis and/or inactivation of T antigen. Such factors might also explain the discrepant results of Tegtmeyer (40) and Brugge and Butel (7). The present study, then, provides no evidence that the early region of the SV40 genome codes either for separate proteins or for one protein with separate active sites. On the other hand, this possibility cannot be excluded. Clarification of this problem should come with the isolation and characterization of SV40 mutants having lesions located in other portions of the early region (25, 38). Of interest in this regard is the discovery by Benjamin (4) of the host range-transformation defective (*hr-t*) mutants of another papovavirus, polyoma. Although the *hr-t* mutation sites map in the early region of the polyoma genome, they lie in a portion of this region distinct from that in which the polyoma *tsA* mutants have been mapped (16, 30). Recently Fluck et al. (17) and Eckhart (15) have demonstrated that the *hr-t* and *tsA* mutants constitute two distinct complementation groups, suggesting that two early genes are present in polyoma.

Finally, mention should be made of the temperature down-shift experiment which demonstrated the reversibility of the thermal inhibition of *tsA*-mediated transformation. In this study, *tsA* transformants exposed to nonpermissive temperature and low serum concentration appeared to resume both DNA synthesis and cell division in a nonrandom fashion on transfer to the permissive temperature, suggesting that at the elevated temperature the majority of cells were blocked at a specific point in the cell cycle. This suggests a method for obtaining cell synchronization which may be useful in a variety of studies of cellular physiology. It should also be noted that this resumption of DNA synthesis and cell growth on transfer from nonpermissive to permissive temperature occurs without the addition of higher concentration of serum. This result differs from that obtained by Martin and Stein (29), who found that the majority of *tsA*-transformed Chinese hamster lung cells did not rapidly resume DNA synthesis on transfer to permissive temperature if they were maintained in media suboptimal for growth of normal cells. The results obtained with BALB/c-3T3 transformants, however, are consistent with the notion that a viral early gene product induces cell DNA synthesis and with the observation that BALB/c-3T3 cells maintained in low concentration of serum can be induced to synthesize DNA on infection with SV40 without the addition of serum (37).

ACKNOWLEDGMENTS

I thank R. G. Martin for stimulating discussions and valuable advice, L. Sadowski for able technical assistance, and W. R. Folk for critical reading of the manuscript.

Portions of this work were initiated at the National Institute of Arthritis, Metabolism and Digestive Diseases while the author was a commissioned officer in the United States Public Health Service. This research was supported by grant no. CA-19816 from the National Cancer Institute.

LITERATURE CITED

1. **Abrahams, P. J., C. Mulder, A. A. van de Voorde, S. O. Warnaar, and A. J. van der Eb.** 1975. Transformation of primary rat kidney cells by fragments of simian virus 40 DNA. J. Virol. **16**:818–823.
2. **Alwine, J. C., S. I. Reed, J. Ferguson, and G. R. Stark.** 1975. Properties of T antigens induced by wild-type SV40 and *tsA* mutants in lytic infection. Cell **6**:529–533.
3. **Anderson, J. L., C. Chang, P. T. Mora, and R. G. Martin.** 1977. Expression and thermal stability of simian virus 40 tumor-specific transplantation antigen and tumor-antigen in wild type- and *tsA* mutant-transformed cells. J. Virol. **21**:459–467.
4. **Benjamin, T.** 1970. Host range mutants of polyoma virus. Proc. Natl. Acad. Sci. U.S.A. **67**:929–934.
5. **Brockman, W. W., M. W. Gutai, and D. Nathans.** 1975. Evolutionary variants of simian virus 40: characterization of cloned complementing variants. Virology **66**:36–52.

6. **Brockman, W. W., and D. Nathans.** 1974. The isolation of simian virus 40 variants with specifically altered genomes. Proc. Natl. Acad. Sci. U.S.A. **71**:942-946.
7. **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.
8. **Carrol, R. B., L. Hager, and R. Dulbecco.** 1974. Simian virus 40 T antigen binds to DNA. Proc. Natl. Acad. Sci. U.S.A. **71**:3754-3757.
9. **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.
10. **Chou, J. Y., and R. G. Martin.** 1974. Complementation analysis of simian virus 40 mutants. J. Virol. **13**:1101-1109.
11. **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.
12. **Crawford, L. V., and D. P. Lane.** 1977. An immune complex assay for SV40 T antigen. Biochem. Biophys. Res. Commun. **74**:323-329.
13. **Danna, K. J., and D. Nathans.** 1971. Specific cleavage of simian virus 40 DNA by restriction endonuclease of *Hemophilus influenzae*. Proc. Natl. Acad. Sci. U.S.A. **68**:2913-2917.
14. **Danna, K. J., G. H. Sack, Jr., and D. Nathans.** 1973. Studies of SV40 DNA. VII. A cleavage map of the SV40 genome. J. Mol. Biol. **78**:363-376.
15. **Eckhart, W.** 1977. Complementation between temperature-sensitive and host range nontransforming mutants of polyoma virus DNA. Virology **77**:589-597.
16. **Feunteun, J., L. Sompayrac, M. Fluck, and T. L. Benjamin.** 1976. Localization of gene functions in polyoma virus DNA. Proc. Natl. Acad. Sci. U.S.A. **73**:4169-4173.
17. **Fluck, M. M., R. J. Staneloni, and T. L. Benjamin.** 1977. Hr-t and ts-A: two early gene functions of polyoma virus. Virology **77**:610-624.
18. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **36**:365-369.
19. **Jessel, D., T. Landau, J. Hudson, T. Lalor, D. Tenen, and D. M. Livingston.** 1976. Identification of regions of the SV40 genome which contain preferred SV40 T antigen-binding sites. Cell **8**:535-545.
20. **Khoury, G., P. Howley, D. Nathans, and M. Martin.** 1975. Posttranscriptional selection of simian virus 40-specific RNA. J. Virol. **15**:433-437.
21. **Kimura, G., and R. Dulbecco.** 1973. A temperature-sensitive mutant of simian virus 40 affecting transforming ability. Virology **52**:529-534.
22. **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.
23. **Kuchino, T., and N. Yamaguchi.** 1975. Characterization of T antigen in cells infected with a temperature-sensitive mutant of simian virus 40. J. Virol. **15**:1302-1307.
24. **Lai, C.-J., and D. Nathans.** 1974. Mapping temperature-sensitive mutants of simian virus 40: rescue of mutants by fragments of viral DNA. Virology **60**:466-475.
25. **Lai, C.-J., and D. Nathans.** 1974. Deletion mutants of simian virus 40 generated by enzymatic excision of DNA segments from the viral genome. J. Mol. Biol. **89**:179-193.
26. **Lai, C.-J., and D. Nathans.** 1975. A map of temperature-sensitive mutants of simian virus 40. Virology **66**:70-81.
27. **MacPherson, I., and L. Montagnier.** 1964. Agar suspension culture for the selective assay of cells transformed by polyoma virus. Virology **23**:291-294.
28. **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.
29. **Martin, R. G., and S. Stein.** 1976. Resting state in normal and simian virus 40 transformed Chinese hamster lung cells. Proc. Natl. Acad. Sci. U.S.A. **73**:1655-1659.
30. **Miller, L. K., and M. Fried.** 1976. Construction of the genetic map of polyoma genome. Virology **18**:824-832.
31. **Osborn, M., and K. Weber.** 1975. Simian virus 40 gene *A* function and maintenance of transformation. J. Virol. **15**:636-644.
32. **Prives, C., E. Gilboa, M. Revel, and E. Winocour.** 1977. Cell-free translation of simian virus 40 early messenger RNA coding for viral T-antigen. Proc. Natl. Acad. Sci. U.S.A. **74**:457-461.
33. **Reed, S. I., J. Ferguson, R. W. Davis, and G. R. Stark.** 1975. T antigen binds to simian virus 40 DNA at the origin of replication. Proc. Natl. Acad. Sci. U.S.A. **72**:1605-1609.
34. **Renger, H. C., and C. Basilico.** 1972. Mutation causing temperature sensitive expression of cell transformation by a tumor virus. Proc. Natl. Acad. Sci. U.S.A. **69**:109-114.
35. **Risser, R., and R. Pollack.** 1974. A nonselective analysis of SV40 transformation of mouse 3T3 cells. Virology **59**:477-489.
36. **Rundell, K., J. K. Collins, P. Tegtmeyer, H. C. Ozer, C.-J. Lai, and D. Nathans.** 1977. Identification of simian virus 40 protein A. J. Virol. **21**:636-646.
37. **Scott, W. A., W. W. Brockman, and D. Nathans.** 1976. Biological activities of deletion mutants of simian virus 40. Virology **75**:319-334.
38. **Shenk, T. E., J. Carbon, and P. Berg.** 1976. Construction and analysis of viable deletion mutants of simian virus 40. J. Virol. **18**:664-671.
39. **Tegtmeyer, P.** 1972. Simian virus 40 deoxyribonucleic acid synthesis: the viral replicon. J. Virol. **10**:591-598.
40. **Tegtmeyer, P.** 1975. Function of simian virus 40 gene *A* in transforming infection. J. Virol. **15**:613-618.
41. **Tegtmeyer, P., and H. J. Ozer.** 1971. Temperature-sensitive mutants of simian virus 40: infection of permissive cells. J. Virol. **8**:516-524.
42. **Tegtmeyer, P., M. Schwartz, J. K. Collins, and K. Rundell.** 1975. Regulation of tumor antigen synthesis by simian virus 40 gene *A*. J. Virol. **16**:168-178.
43. **Tenen, D. G., P. Baygell, and D. Livingston.** 1975. Thermolabile T (tumor) antigen from cells transformed by a temperature-sensitive mutant of simian virus 40. Proc. Natl. Acad. Sci. U.S.A. **72**:4351-4355.
44. **Tenen, D. G., R. G. Martin, J. Anderson, and D. M. Livingston.** 1977. Biological and biochemical studies of cells transformed by simian virus 40 temperature-sensitive gene *A* mutants and *A* mutant revertants. J. Virol. **22**:210-218.