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Differential Ability of a T-Antigen Transport-Defective Mutant of Simian Virus 40 To Transform Primary and Established Rodent Cells

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The transforming potential and oncogenicity of a simian virus 40 (SV40) mutant affecting T-antigen (T-ag), SV40(cT)-3, was examined in an effort to dissect T-ag functions in transformation. SV40(cT)-3 has a point mutation at nucleotide 4434 that abolishes the transport of T-ag to the nucleus but does not affect its association with the cell surface. Transfection-transformation assays were performed with primary cells and established cell lines of mouse and rat origin. The efficiency of transformation for established cell lines by SV40(cT)-3 was comparable to that of wild-type SV40, indicating that transformation of established cell lines can occur in the absence of detectable amounts of nuclear T-ag. Transformation of primary mouse embryo fibroblasts by SV40(cT)-3 was markedly influenced by culture conditions; the relative transforming frequency was dramatically reduced in assays involving focus formation in low serum concentrations or anchorage-independent growth. Immunofluorescence tests revealed that the transformed mouse embryo fibroblasts partially transport the mutant cT-ag to the cell nucleus. Transformed cell lines induced by SV40(cT)-3 did not differ in growth properties from wild-type transformants. SV40(cT)-3 was completely defective for the transformation of primary baby rat kidney cells, a primary cell type unable to transport the mutant T-ag to the nucleus. The intracellular localization of cellular protein p53 was found to mimic T-ag distribution in all the transformants analyzed. The mutant virus was weakly oncogenic in vivo: the induction of tumors in newborn hamsters by SV40(cT)-3 was reduced in incidence and delayed in appearance in comparison to wild-type SV40. These observations suggest that cellular transformation is regulated by both nuclear and surface-associated forms of SV40 T-ag.

Simian virus 40 (SV40) is an oncogenic DNA virus capable of transforming cells in tissue culture and inducing tumors in newborn hamsters. The mechanism of transformation by SV40 has been investigated extensively but has not been completely elucidated. The SV40 A gene has been implicated in both the initiation and maintenance of the transformed state by studies of temperature-sensitive mutants of the A gene (2, 3, 17, 28, 31, 47, 48). Although temperature-insensitive transformants can be isolated after transformation by SV40 tsA mutants, this phenomenon is poorly understood and is dependent on both the growth conditions of the cells at the time of transformation and the assay used to measure transformation (34, 35, 41). The entire A gene encodes the SV40 large tumor antigen (T-ag), with some of the DNA sequences being used to direct the synthesis of small t-ag (6, 11, 36). T-ag is localized in both the nucleus and plasma membrane (8, 9, 20, 24, 40, 45, 46), with nuclear T-ag representing more than 95% of the total cellular T-ag (24, 45). The transforming activity of SV40 has been generally presumed to reside in the nuclear fraction of T-ag, with no definitive function ascribed to cell-surface-associated T-ag. Small t-ag does not play an indispensible role in SV40 transformation but may be required for the transformation of cells in a resting state (29, 42, 43).

The early region of polyomavirus is organized differently, encoding three products, large, middle, and small tumor antigen (12, 44). The large T-ag is localized in the nucleus, whereas the middle T-ag is found on the cell surface (15). The middle T-ag is capable of transforming established cell lines by itself but requires the aid of either large T-ag or small t-ag for transformation of primary cells and induction of tumors in newborn rats (1, 33). Since SV40 T-ag is localized both in the nucleus and on the cell surface, the potential exists for that single protein to provide the functions represented individually by the polyomavirus large T-ag and middle T-ag. Transformation of primary cells by oncogenes isolated from human tumors and RNA tumor viruses may also require multiple, complementary events. Transformation of primary baby rat kidney (BRK) cells by the *ras* gene (a plasma membrane protein) was achieved only with the assistance of the *myc*, adenovirus E1A, or polyomavirus large T-ag gene (all nuclear proteins) (18, 39).

To demonstrate a possible dual functionality for SV40 T-ag in transformation, mutants are required which specifically affect nuclear or cell surface T-ag. An SV40 mutant which affects the appearance of T-ag in the nucleus but not in the plasma membrane has been described. A mutant of the SV40-adenovirus 7 hybrid virus, PARA, was isolated that induces SV40 T-ag which is not transported to the nucleus and accumulates in the cytoplasm (4, 21, 22). The cT mutation does not inhibit the localization of T-ag on the cell surface (24). Initial investigations of the PARA(cT) mutant suggested that its transforming capacity relative to wild-type (WT) virus was dramatically reduced (10, 23, 32, 37). Recently, the SV40 sequences from the PARA(cT) hybrid virus were used to construct an intact SV40 genome containing the cT mutation (25). The mutant phenotype is due to a single amino acid change at amino acid 128. A positively charged lysine in WT T-ag is replaced by a neutral asparagine in the mutant T-ag, which interrupts a stretch of five positively charged amino acids. Similar mutants have been created by mutagenesis of that coding region of the SV40 genome (16). It has been speculated that the five positively charged amino acids may represent a receptor site or "sig-

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nal" required for the transport of T-ag to the nucleus (16, 25).

This report describes an investigation of the transforming potential of SV40(cT)-3. Transformation was examined in established and primary cells, and oncogenicity was determined in newborn hamsters. The results suggest that transformation of established cell lines by SV40(cT)-3 is not significantly reduced under normal culture conditions, that transformation of primary cells does not occur in the absence of detectable levels of nuclear T-ag, and that induction of tumors by SV40(cT)-3 is reduced and delayed in comparison with that by WT SV40. Together, these observations suggest that both nuclear and surface-associated forms of T-ag are functionally important in cellular transformation.

MATERIALS AND METHODS

Cells. The BALB/c-3T3E cell line was a gift from William Brockman (2), and the normal rat kidney cell line (NRK-49F) (7), obtained originally from the American Type Culture Collection (ATCC CRL-1570), was provided by Lynn Yeoman. Primary BALB/c mouse embryo fibroblasts (MEF) were prepared from 10- to 12-day-old BALB/c embryos, and primary BRK cells were derived from 9- to 16-day-old Sprague-Dawley rats. All cells were grown in Dulbecco modified minimal essential medium (GIBCO Laboratories, Grand Island, N.Y.), supplemented with 10% fetal bovine serum (FBS; GIBCO), 25 μg of gentamicin sulfate (Schering Corp., Bloomfield, N.J.) per ml, and 0.3% sodium bicarbonate.

Plasmids. The bacterial plasmid pBR322 was a generous gift of James Pipas. Plasmid pBSV-1 is an intact copy of the WT SV40 genome cloned into pBR322 at the *Bam*HI site (25). Plasmid pBSVcT-3 is an intact copy of the SV40(cT)-3 genome cloned into pBR322 at the *Bam*HI site (25). SV40(cT)-3 has a point mutation at nucleotide 4434 that abolishes the transport of T-ag to the nucleus (25). Plasmids were purified from cultures of *Escherichia coli* HB101 after overnight amplification in chloramphenicol. Plasmid DNA was prepared by an alkaline extraction procedure and hydroxylapatite chromatography as described previously (25). SV40 sequences represent 54.58% of each recombinant plasmid.

Transfections. Plasmid DNA was linearized by digestion with restriction endonuclease *Eco*RI (Bethesda Research Laboratories, Bethesda, Md.), and calcium phosphate precipitates were formed as described previously (13). Cell cultures growing in 60-mm dishes were exposed to 5 μg of precipitated DNA for 30 min at 37°C. The cultures were flooded with 4 ml of Dulbecco modified minimal essential medium containing 100 μM chloroquine (27) and incubated at 37°C for 4 h, at which time the cultures were given a 15% glycerol shock for 3 min at 37°C. Cell cultures were washed twice with Dulbecco modified minimal essential medium and incubated overnight in 5 ml of medium. The following day each 60-mm plate was subcultured into 6 to 18 (depending on cell type) 100-mm dishes. Cultures were incubated at 37°C, refed twice weekly, and stained with hematoxylin 21 days posttransfection. Alternatively, cells were seeded in medium containing 1.2% methylcellulose (as described below) the day after transfection, and colonies were counted 21 days posttransfection.

Induction of tumors in hamsters. Within 24 h of birth Syrian golden hamsters (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) were injected subcutaneously on the back of the neck with 5 μg of plasmid DNA. All plasmids were linearized by digestion with restriction endonuclease *Eco*RI before injection. Baby hamsters were weaned 3 weeks after birth. Tumors which appeared were surgically excised, trypsinized, and cultured. The intracellular localization of T-ag in cultured tumor cell lines was assessed by immunofluorescence (IF).

IF. The IF staining of SV40 T-ag was performed on acetone-fixed (3-min) monolayers with SV40 tumor-bearing hamster serum (hamster ascites fluid) (20) as previously described (32). The IF staining of the cellular protein p53 was accomplished as above with monoclonal antibody PAb421 (14).

Saturation density. Cells were seeded at a density of 2×10^5 cells per 35-mm dish in medium supplemented with 10 or 1% FBS. Cell counts were performed on triplicate plates every day, and cultures were refed every other day for the duration of the experiment (5).

Colony formation in methylcellulose. Colony formation in methylcellulose was performed by a modification (5) of the method of Risser and Pollack (38). Cells were seeded at a density of 10^3 and 10^5 cells per 60-mm dish for established cell lines and transfected cultures, respectively. Methylcellulose medium consisted of Dulbecco modified minimal essential medium supplemented with 10 or 1% FBS, 1.2% methylcellulose (4,000 cps; Fisher Scientific Co., Pittsburgh, Pa.), 50 μg of gentamicin sulfate per ml, and 0.3% sodium bicarbonate. The methylcellulose medium containing the cells was placed on top of a basal agar layer in which 0.9% agar was substituted for methylcellulose in the above medium. Cultures were refed on days 7 and 14, and colonies were counted on day 21.

RESULTS

Transforming potential of SV40(cT)-3 in mouse cells. The transforming potential of SV40(cT)-3 was compared to that of WT virus in a continuous cell line and in primary cells of mouse origin. BALB/c-3T3 cells served as the immortalized cell recipients, and MEF were used as primary cells. Analyses used plasmid DNA because SV40(cT)-3 virus stocks produced in COS-1 cells contain a low level of WT virus due to recombination of the mutant genome with the resident integrated copy of SV40 DNA (25). Transformation assays were performed with 5 μg of plasmid DNA per 60-mm culture. Cells were incubated with calcium phosphate precipitates in the presence of 100 μM chloroquine and then were given a 15% glycerol shock as described above. The cultures were incubated overnight in growth medium, and then either each plate of cells was split into six 100-mm plates or the cells were seeded into methylcellulose. Transformed foci and anchorage-independent colonies were scored 21 days after transfection. Only darkly stained, well-defined foci on plastic and large colonies in methylcellulose were counted. Final counts were adjusted to reflect transforming events per microgram of SV40 DNA (contained in the plasmid inocula). Transfection with control pBR322 DNA did not result in the appearance of any foci or colonies in our assays.

The transformation efficiency of 3T3 cells, as judged by foci formation on plastic, was approximately equivalent for pBSVcT-3 and pBSV-1 (the WT SV40 plasmid). Similarly, the transforming frequency by pBSVcT-3 of MEF on plastic was reduced about 2.5 times compared with that by pBSV-1 (Table 1). The ability of pBSVcT-3 to induce 3T3 cells to form colonies in methylcellulose was reduced about twofold compared with that of pBSV-1. It is noteworthy that the transforming frequency of primary MEF by both plasmids, as measured by anchorage-independent growth, was mark-

TABLE 1. Transforming potential of SV40(cT)-3 in mouse cells measured in two types of assays[a]

Cell line	Plasmid	Transforming frequency on:			
		Plastic		Methylcellulose	
		Foci/μg of SV40 DNA	Ratio (WT/cT)	Colonies/ μg of SV40 DNA	Ratio (WT/cT)
3T3	pBSV-1	201	1.0	122	2.0
	pBSVcT-3	194		62	
MEF	pBSV-1	168	2.5	8.15	11.0
	pBSVcT-3	68		0.74	

[a] Cultures of BALB/c-3T3 cells and primary MEF were transfected with plasmid DNA (5 μg) as described in the text. Transfected cells were examined for the ability to form foci on plastic and colonies in methylcellulose semisolid medium. The ratios of foci and colonies induced per microgram of SV40 DNA by the WT SV40 plasmid, pBSV-1, and the SV40(cT)-3 mutant plasmid, pBSVcT-3, are indicated.

edly reduced when compared with focus formation on plastic. However, it is evident that the transforming ability of the mutant pBSVcT-3 was deficient in MEF under these conditions, being 11-fold lower relative to that of pBSV-1 (Table 1).

Next, we examined the ability of the cT mutant to transform mouse cells cultured on plastic in low FBS concentrations. Transfections were performed with the same conditions as described above; on the next day, each transfected culture was split into six 100-mm plates in media containing either 10 or 1% FBS. When this protocol was used, the ability of pBSVcT-3 to transform 3T3 cells grown in either high (10%) or low (1%) FBS was only slightly reduced when compared with that of pBSV-1 (Table 2). Transformation of MEF maintained in 10% FBS was also slightly lower (2.3-fold) for pBSVcT-3 than WT virus. However, transformation of MEF cells by the cT mutant was substantially lower (10-fold) than for WT virus when the cells were cultured in 1% FBS.

The results of these assays suggest that the efficiency of transformation of an immortalized cell by SV40(cT)-3 is equivalent to that by WT SV40 under standard culture conditions on plastic. When more stringent culture conditions are used, such as growth in the presence of low FBS concentrations or in semisolid medium, the transformation of immortalized cells by SV40(cT)-3 is still only marginally reduced. Importantly, the transforming frequency of primary cells by SV40(cT)-3 is approximately 10-fold lower than that for WT when the recipient cells are grown under stringent culture conditions.

TABLE 2. Effect of FBS concentration on focus formation by SV40(cT)-3 in mouse cells[a]

Cell line	FBS (%)	Plasmid	Foci/μg of SV40 DNA	Ratio (WT/cT)
3T3	10	pBSV-1	262	1.7
		pBSVcT-3	159	
	1	pBSV-1	110	2.1
		pBSVcT-3	52	
MEF	10	pBSV-1	108	2.3
		pBSVcT-3	48	
	1	pBSV-1	67	10.0
		pBSVcT-3	6.7	

[a] Cultures of BALB/c-3T3 cells and primary MEF were transfected with plasmid DNA (5 μg) as described in the text. Transfected cells were examined for the ability to form foci on plastic in medium containing 10 or 1% FBS. The ratios of foci induced by the WT SV40 plasmid, pBSV-1, and the SV40(cT)-3 mutant plasmid pBSVcT-3 are indicated.

IF localization of T-ag in SV40(cT)-3-transformed mouse cells. To further characterize the SV40(cT)-3 mouse transformants, five foci or colonies from each of the transformation conditions described above were selected, and transformed cell lines were established. Each colony was examined by IF for the intracellular localization of T-ag. All of the cell lines transformed by WT SV40 displayed the typical nuclear T-ag pattern of fluorescence (Fig. 1A and C). The 3T3 cells transformed by SV40(cT)-3 displayed a bright cytoplasmic reaction for T-ag (Fig. 1B). In addition, a very faint, diffuse nuclear reaction was observed in the SV40(cT)-3-transformed 3T3 cells; this reaction was not distinguishable from the faint background reaction obtained with the hamster anti-T serum on normal 3T3 cells. In contrast, all of the MEF cells transformed by SV40(cT)-3 exhibited both cytoplasmic and nuclear fluorescence for T-ag. The nuclear reactivity in MEF cells transformed by SV40(cT)-3 was judged to be specific due to its intensity and to the distinctive granular staining pattern typical for T-ag (Fig. 1D). The stringency of culture conditions had no observable effect on the ratio of nuclear to cytoplasmic staining for T-ag in MEF cells transformed by SV40(cT)-3.

These results demonstrated that transformation of immortalized mouse cells occurred in the absence of detectable accumulation of nuclear T-ag and that primary MEF cells transformed by SV40(cT)-3 were capable of transporting some mutant T-ag polypeptides to the nucleus.

Since the efficiency of transformation of MEF cells by pBSVcT-3 and pBSV-1 under low-stringency culture conditions was comparable, it is unlikely that the transport of mutant T-ag by MEF cells is due to reversion of the mutation or to the selection of a rare cell type within the MEF population capable of nuclear transport of mutant T-ag. To determine whether the transport of T-ag was due to an adaptation process that occurred after initiation of transformation or to an inherent property of many MEF cells, we examined MEF cells 48 h after infection with SV40(cT)-3 virus (25) for the intracellular distribution of T-ag. The IF assays revealed that within 48 h about 50% of the cells expressing cytoplasmic T-ag already exhibited some T-ag in the nucleus (data not shown). In contrast, infection of TC-7 cells with the same SV40(cT)-3 virus preparation resulted in less than 0.5% of the cells exhibiting any nuclear T-ag, a marker previously shown to reflect the level of WT SV40 recombinants present in mutant virus stocks prepared in COS-1 cells (25). Therefore, the amount of WT SV40 contaminant in this SV40(cT)-3 virus preparation could not account for the high percentage of MEF cells expressing nuclear T-ag soon after infection with SV40(cT)-3. These results suggest that some primary mouse embryonic cells are capable of partial nuclear transport of the mutant T-ag and that the phenotypic transformants arise from that subset of cells.

IF localization of p53 in SV40(cT)-3-transformed mouse cells. The association of T-ag with a host-encoded protein designated p53 has been well documented (19, 26, 30). It was of interest to determine the intracellular distribution of p53 in transformed cells expressing a cytoplasmic T-ag. Each of the transformed cell lines described above was examined by IF with the monoclonal antibody PAb421, which is specific for p53 (14, 40). In each instance, the distribution of p53 mimicked the IF reaction obtained for T-ag. All cells transformed by WT SV40 exhibited a nuclear reaction for p53 (Fig. 2A and C), 3T3 cells transformed by SV40(cT)-3 had a cytoplasmic reaction for p53 (Fig. 2B); and MEF cells transformed by SV40(cT)-3 displayed p53 in both the cyto-

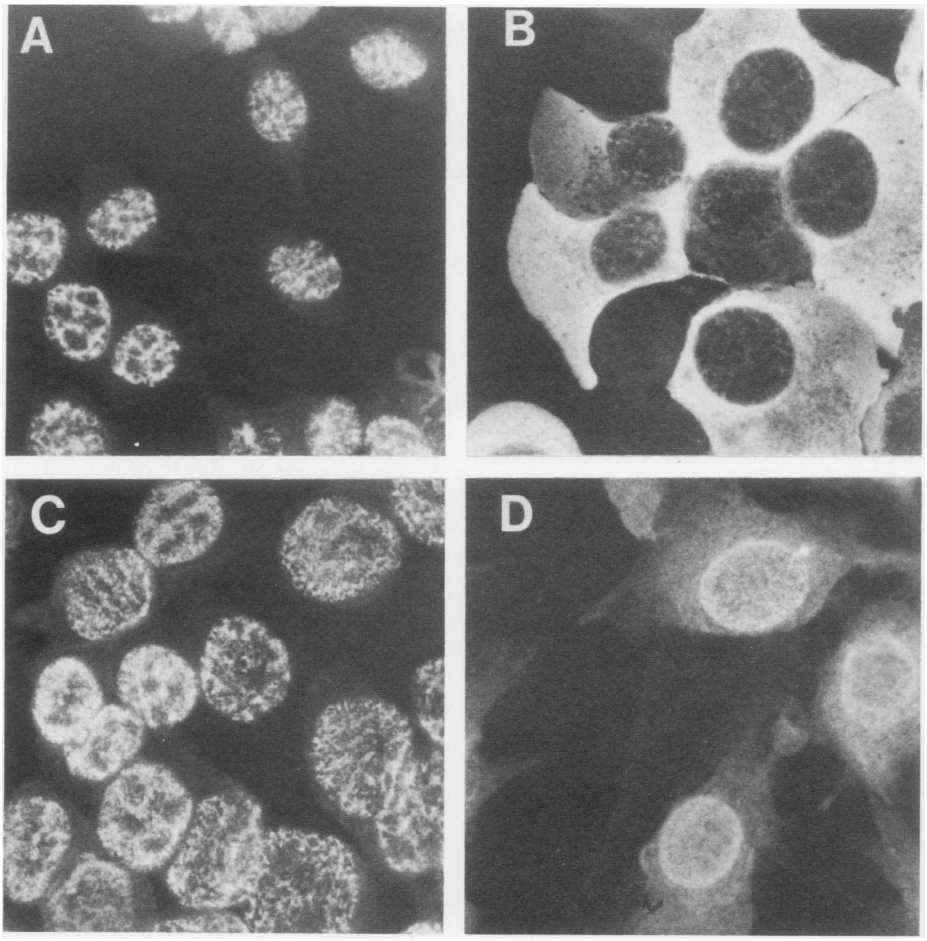

FIG. 1. Intracellular distribution of SV40 T-ag in transformed mouse cells. BALB/c-3T3 cells and primary MEF transformed by WT SV40 or SV40(cT)-3 were examined by using hamster anti-T sera (hamster ascites fluid) as described in the text. A, BALB/c-3T3 cells transformed by WT SV40; B, BALB/c-3T3 cells transformed by SV40(cT)-3; C, MEF transformed by WT SV40; D, MEF transformed by SV40(cT)-3. Note nuclear fluorescence as well as cytoplasmic reactivity in panel D. Magnification, ×500.

plasm and the nucleus (Fig. 2D). These results suggest that the association with T-ag is the determining factor regulating the intracellular localization and accumulation of p53 to levels detectable by IF in rodent cells transformed by SV40.

Growth properties of SV40(cT)-3-transformed mouse cells. The difference in distribution of SV40 T-ag and p53 in the transformed MEF and BALB/c-3T3 cells induced by WT SV40 or the cT mutant raised the intriguing question of possible effects on the growth properties of those cells. Several clones of cells derived from foci on plastic and characterized by IF for distribution of T-ag were randomly selected for growth analyses. Both saturation density on plastic and colony formation in methylcellulose were assayed (Table 3). There were pronounced ranges among the clones evident in both assays, but the cT transformants did not appear to be generally less phenotypically transformed than the WT transformants. All the 3T3 transformants tended to grow to higher saturation densities than the transformed MEF cells. There were marked variabilities among the anchorage-independent growth abilities of the clonal lines, a phenomenon observed in previous studies (5, 38).

It is evident from the clonal lines analyzed that 3T3 cells expressing T-ag only in the cytoplasm and on the surface are as phenotypically transformed as 3T3 cells with T-ag localized both in the nucleus and on the surface. The MEF cells able to partially transport mutant cT-ag to the nucleus are also as phenotypically altered as those transformed by WT virus.

Transforming potential of SV40(cT)-3 in rat cells. The ability of SV40(cT)-3 to transform cells of a continuous line as well as primary cells of rat origin was next examined. The established rat cell line used was NRK (a cell line derived from normal rat kidney), whereas primary cells were obtained from BRKs. The procedure for transfection of cells with plasmid DNA was identical to the conditions described above for mouse cells, except that transformation was measured only by focus formation on plastic in the presence of 10% FBS. The transformation efficiency of pBSVcT-3 on NRK cells was reduced about twofold in comparison with that of pBSV-1 (Table 4). These results are comparable to those obtained when immortalized mouse cells were used (Tables 1 and 2). However, when transformation of primary BRK cells was attempted, pBSVcT-3 was incapable of inducing any detectable foci in two separate experiments (Table 4). These failures were not due to any natural resistance on the part of the primary BRK cells since transfection with WT SV40 plasmid pBSV-1 resulted in the formation of well-defined, dense foci at an efficiency equivalent to that observed with the other cell types.

These results indicate that SV40(cT)-3 is not capable of transforming primary BRK cells. Since the transformation of

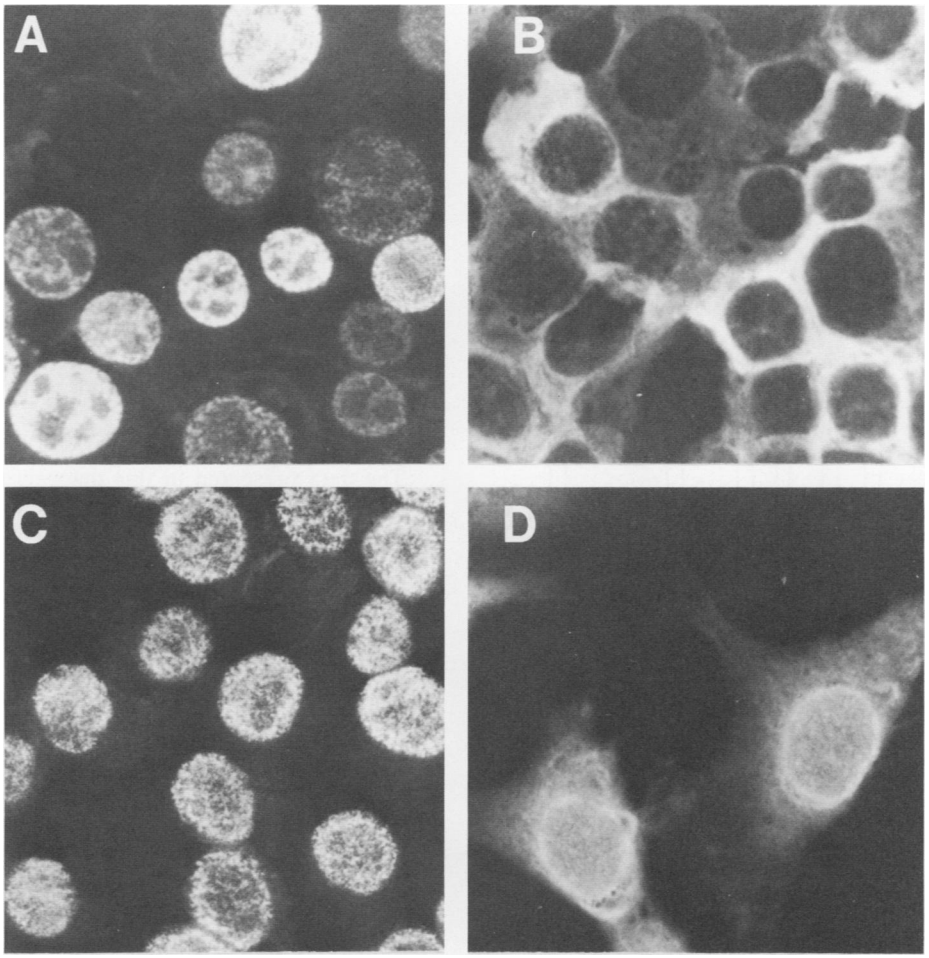

FIG. 2. Intracellular distribution of cellular protein p53 in transformed mouse cells. BALB/c-3T3 cells and primary MEF transformed by WT SV40 or SV40(cT)-3 were examined by IF with monoclonal antibody PAb421. A, BALB/c-3T3 cells transformed by WT SV40; B, BALB/c-3T3 cells transformed by SV40(cT)-3; C, MEF transformed by WT SV40; D, MEF transformed by SV40(cT)-3. Note that the intracellular distribution of p53 parallels that of SV40 T-ag shown in Fig. 1. Magnification, ×500.

TABLE 3. Growth properties of clonal lines of SV40 WT- and SV40(cT)-3-transformed MEF and BALB/c-3T3 cells[a]

Cell line	Clone	T-ag[b]		Cell density [(cells/cm^2) × 10^5]		Ratio (10%/1%)	Colony-forming ability (%)	
		Nuc	Cyt	10% FBS	1% FBS		10% FBS	1% FBS
MEF/SV-1/10	2	+	0	4.6	2.2	2.1	41.0	12.8
	4	+	0	8.4	2.3	3.7	37.5	0.4
MEF/SV-1/1	1	+	0	4.8	3.1	1.5	0.6	<0.1
	3	+	0	2.8	1.3	2.2	3.8	0.1
MEF/cT-3/10	1	+	+	4.1	2.3	1.8	15.5	1.9
	3	+	+	3.1	0.7	4.4	16.6	2.6
MEF/cT-3/1	1	+	+	2.1	0.7	3.0	6.4	<0.1
	2	+	+	2.5	0.9	2.8	<0.1	<0.1
3T3/SV-1/10	1	+	0	5.1	4.0	1.3	0.9	<0.1
	2	+	0	5.3	4.1	1.3	0.9	0.2
3T3/SV-1/1	1	+	0	6.2	5.3	1.2	1.5	0.5
	2	+	0	5.7	5.5	1.0	<0.1	<0.1
3T3/cT-3/10	1	0	+	5.6	3.2	1.8	2.2	<0.1
	2	0	+	5.3	2.8	1.9	1.2	0.3
3T3/cT-3/1	2	0	+	4.2	4.0	1.0	<0.1	<0.1
	3	0	+	5.6	4.3	1.3	0.2	<0.1

[a] Foci were picked from transformation assays, and cell lines were established. The designation of each cell line indicates the cell type (MEF or 3T3), the transforming plasmid (SV-1 or cT-3), and the FBS concentration (10 or 1%) used in the original transformation assay. The clonal lines were assayed for intracellular distribution of T-ag by IF and for growth properties (saturation density on plastic and colony-forming ability in methylcellulose) as described in the text.

[b] Nuc, Nuclear; Cyt, cytoplasmic.

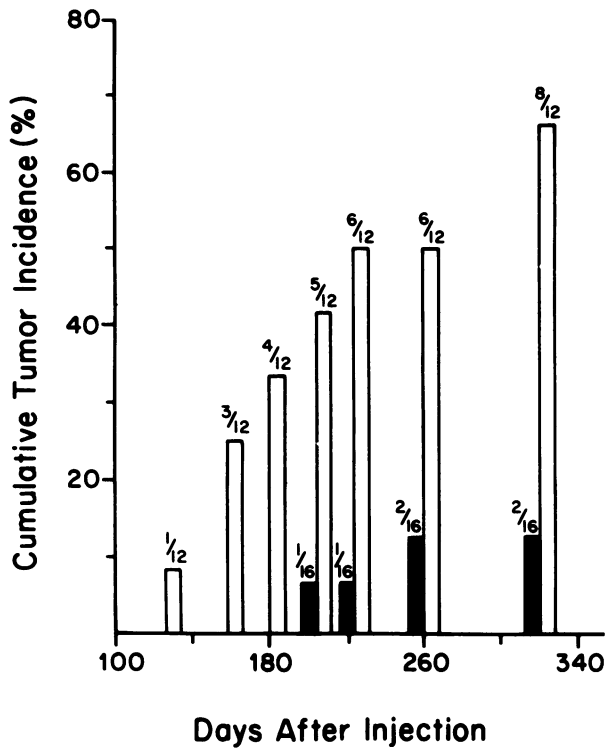

FIG. 3. Oncogenicity of SV40(cT)-3 and WT SV40 in newborn hamsters. Plasmid DNA (5 μg) was inoculated subcutaneously into the neck of newborn hamsters. Injected animals were observed for a period of 1 year for tumor formation. The data are presented as the cumulative tumor incidence; open and closed bars represent animals injected with pBSV-1 and pBSVcT-3, respectively. The ratio of the number of animals that developed tumors to the total number of animals injected is presented at the top of each bar.

primary MEF cells by SV40(cT)-3 was associated with the capacity of some MEF cells to partially transport the mutant T-ag to the nucleus, it was necessary to determine whether any primary BRK cells were capable of transporting cT-ag to the nucleus. Primary BRK cells were infected with SV40(cT)-3 virus, harvested, and tested by IF at 48 h postinfection. Approximately 30% of the BRK cells expressed cytoplasmic T-ag by IF; no detectable nuclear staining was observed (data not shown). These results imply that the capacity of SV40(cT)-3 to transform primary cells is dependent on the ability of certain primary cells to transport some mutant T-ag to the nucleus.

Oncogenicity of SV40(cT)-3 in newborn hamsters. The determination of transforming potential of a given gene by the use of in vitro transformation assays is subject to considerable variation depending on the cell type and the growth conditions used for the assay. A direct correlation between a single in vitro assay and oncogenicity in vivo has not been demonstrated. The oncogenicity of SV40(cT)-3 in newborn hamsters was examined because of its reduced transforming potential apparent in in vitro assays under stringent culture conditions. Newborn hamsters were injected with pBSV-1 or pBSVcT-3 plasmid DNAs as described above, and the hamsters were observed for tumor development for a period of 12 months. WT SV40 plasmid pBSV-1 induced tumors in 66.6% (8/12) of the recipient animals, whereas the frequency of tumor formation for pBSVcT-3 was only 12.5% (2/16) (Fig. 3). In addition, the latent period for tumor formation was considerably longer for pBSVcT-3–induced tumors. The earliest arising tumors appeared 134 and 206 days after inoculation with pBSV-1 and pBSVcT-3, respectively. These data are consistent with the weakly oncogenic nature of the parental PARA(2cT)-adenovirus 7 hybrid virus which induced only one tumor in 24 newborn hamsters (37).

Cell lines were established from the two tumors induced by pBSVcT-3 to determine the intracellular localization of T-ag by IF. Both tumor lines displayed an intense cytoplasmic reaction for T-ag; one tumor line exhibited some weak nuclear fluorescence, whereas the other had no detectable nuclear reactivity.

These results demonstrate that SV40(cT)-3 is capable of inducing tumors in newborn hamsters, albeit at a markedly reduced frequency and with an extended latent period before tumor appearance in comparison with WT virus.

DISCUSSION

The data presented in this manuscript demonstrate that SV40(cT)-3, a mutant defective for the nuclear transport of T-ag, represents a new class of SV40 mutant with respect to transformation. In our interpretation of these data, we assumed that the differences in the transforming potential of SV40(cT)-3 compared with that of WT SV40 are a result of the defect in the nuclear transport of T-ag and are not due to an unknown effect of the mutation at amino acid 128. This assumption is supported in part by the fact that cT-ag still has the capacity to bind to cellular DNA, to the origin of viral DNA synthesis, and to the cellular protein p53 (22; this manuscript; unpublished data).

SV40(cT)-3 was capable of transforming established mouse and rat cell lines at an efficiency comparable to that of WT SV40. IF assays revealed that transformation of immortalized cells occurred in the absence of detectable levels of nuclear T-ag. SV40(cT)-3 also had the capacity to transform primary MEF cells, although in all of those transformants examined cT-ag was partially transported to the nucleus. In addition, culture conditions had a dramatic effect on the efficiency of transformation of MEF cells by SV40(cT)-3, with low FBS concentrations or suspension in semisolid medium sharply reducing the frequency of transformation events. In contrast, SV40(cT)-3 was completely transformation defective when assayed on primary BRK cells, a cell type incapable of transporting detectable levels of cT-ag to the nucleus.

At least two plausible explanations can be presented for the transformation characteristics of SV40(cT)-3. In one

TABLE 4. Transforming potential of SV40(cT)-3 in rat cells measured by focus formation[a]

Cell line	Plasmid	Foci/μg of SV40 DNA	Ratio (WT/cT)
NRK	pBSV-1	111.8	2.0
	pBSVcT-3	54.6	
BRK			
Expt 1	pBSV-1	12.8	
	pBSVcT-3	0	
Expt 2	pBSV-1	107.1	
	pBSVcT-3	0	

[a] Cultures of established NRK cells and primary BRK cells were transfected with plasmid DNA (5 μg) as described in the text. Transfected cells were assayed for the ability to form foci on plastic. The ratio of foci induced by the WT SV40 plasmid pBSV-1 and the SV40(cT)-3 mutant plasmid pBSVcT-3 is indicated.

model, transformation would be regulated exclusively by nuclear T-ag. It would be assumed that the cT mutation dramatically reduces the transport of T-ag to the nucleus but does not abolish it completely. This model suggests that primary cells require more nuclear T-ag than do immortalized cells to exhibit transformed properties and that the stringency of the culture conditions also influences the level of T-ag required for the expression of transformation. A second hypothesis would propose that transformation is regulated by both nuclear and plasma membrane-associated T-ag. In this model, immortalized cells could dispense with the nuclear T-ag function (except possibly under stringent culture conditions whereby the cellular immortalizing function might be insufficient to promote continued cellular proliferation). This model predicts that the cT mutant would be transformation negative in primary cells. Transformation of MEF cells by SV40(cT)-3 is an exception under either model due to the partial transport of cT-ag to the nucleus.

Currently, we favor the latter hypothesis. Although the possibility cannot be completely excluded that an undetectable yet biologically functional amount of cT-ag is transported to the nucleus in each transformed cell, several considerations support the concept of a role for membrane-associated T-ag in transformation. The cT mutation that abolishes the transport of T-ag to the nucleus does not abolish the association of cT-ag with the plasma membrane (24). The majority of intracellular cT-ag is present in the soluble cytoplasm (22, 24), the level of cell surface-associated T-ag is elevated in comparison to WT T-ag (24), and cT-ag is not detectable in the nucleus by IF (4, 21, 25, 32). The amount of surface-associated T-ag appears to correlate with cell growth of SV40-transformed mouse cells; much higher levels are detected on actively growing cells than on populations of resting cells, suggesting a possible role for surface T-ag in cell growth (40a). Furthermore, microinjection of plasmid DNA into quiescent cells showed that SV40(cT)-3 is able to stimulate cellular DNA synthesis as efficiently as is WT SV40 (R. E. Lanford, J. K. Hyland, R. Baserga, and J. S. Butel, Mol. Cell. Biol., in press). This suggests that surface-associated T-ag is involved in stimulation of cellular DNA synthesis and that the cT mutation that affects nuclear transport does not adversely affect the membrane function. The alternate hypothesis [i.e., that the transforming capacity of SV40(cT)-3 is due to a low level of nuclear T-ag] would infer that sufficient nuclear T-ag is present in each cT-ag-positive cell for transformation to be maintained but not for growth of the cells under stringent conditions. Yet, no correlation was observed between the presence of nuclear T-ag and the ability of clonally derived transformants to grow under stringent culture conditions (Table 3). Finally, in the closely related polyomavirus system, both the nuclear large T-ag and the plasma-membrane-associated middle T-ag (15) play important roles in transformation (1, 33).

Two observations made during the course of this investigation are significant with regard to the mechanism by which proteins are transported to the nucleus. The intracellular localization of p53 mimicked the distribution of T-ag in all transformed cells. These results indicate that the cT mutation inhibits the transport of p53, presumably due to the formation of T-ag–p53 complexes. Interestingly, the cT mutation inhibits the nuclear transport of WT T-ag in cells synthesizing both cT-ag and WT T-ag (21, 25). One of the proposed mechanisms for this phenomenon is the coaggregation of cT-ag and WT T-ag and inhibition of transport of the entire complex due to the cT-ag component of the oligomer. Thus, the cT mutation may be capable of inhibiting, or greatly diminishing, the transport of any protein that forms a stable complex with T-ag.

The second important observation is that a subpopulation of cells present in primary MEF cultures has the capacity to partially transport cT-ag to the nucleus. This implies that some cells, possibly of certain embryonic origin, are more permissive in their recognition of the sequence in T-ag required for nuclear transport, such that the mutated sequence in cT-ag is partially recognized. Previous studies have demonstrated that during extensive subculturing cells able to partially transport the mutant T-ag arise spontaneously from clonally derived transformed cells and from tumor lines that originally expressed only cytoplasmic T-ag (23, 37). The critical physiological or biochemical difference between cells able to partially transport cT-ag and cells that do not remains to be elucidated.

The induction of tumors in newborn hamsters by SV40(cT)-3, albeit at a reduced frequency and with an extended latent period (37; this report), appears to conflict with the inability of the mutant to transform primary BRK cells. However, Bouchard and co-workers (1) recently demonstrated that polyomavirus middle T-ag alone was able to induce tumors in newborn hamsters but was incapable of transforming primary rat cells in vitro or of inducing tumors in newborn rats. These data suggest that virus-mediated oncogenicity is considerably more complex than most simplistic models assume and that the in vitro transformation systems available do not adequately represent the in vivo situation.

The results presented within this report are highly suggestive that transformation of primary cells by SV40 is dependent upon both nuclear and surface-associated forms of T-ag. The postulated bifunctional nature of SV40 transformation is reminiscent of recent observations made with polyomavirus and cellular oncogenes. Polyomavirus large T-ag, *myc*, and adeno virus E1A functions can complement *ras* and polyomavirus middle T-ag to accomplish transformation of primary rat cells (18, 39). It is conceivable that transformation of primary cells by other viral agents also requires the cooperation of nuclear and nonnuclear functions. Studies are underway to determine whether other genes capable of immortalizing cells can complement SV40(cT)-3 for transformation of primary BRK cells. Successful complementation would greatly strengthen the hypothesis that surface-associated SV40 T-ag provides a crucial function in cellular transformation.

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LITERATURE CITED

1. **Bouchard, L., C. Gelinas, C. Asselin, and M. Bastin.** 1984. Tumorigenic activity of polyoma virus and SV40 DNAs in newborn rodents. Virology **135**:53–64.
2. **Brockman, W. W.** 1978. Transformation of BALB/c-3T3 cells by *ts*A mutants of simian virus 40: temperature sensitivity of the transformed phenotype and retransformation by wild-type virus. J. Virol. **25**:860–870.
3. **Brugge, J. S., and J. S. Butel.** 1975. Role of simian virus 40 gene A function in maintenance of transformation. J. Virol. **15**:619–635.
4. **Butel, J. S., M. J. Guentzel, and F. Rapp.** 1969. Variants of defective simian papovavirus 40 (PARA) characterized by cytoplasmic localization of simian papovavirus 40 tumor antigen. J. Virol. **4**:632–641.
5. **Butel, J. S., C. Wong, and D. Medina.** 1984. Transformation of

mouse mammary epithelial cells by papovavirus SV40. Exp. Mol. Pathol. **40**:79–108.
6. **Crawford, L. V., C. N. Cole, A. E. Smith, E. Paucha, P. Tegtmeyer, K. Rundell, and P. Berg.** 1978. Organization and expression of early genes of simian virus 40. Proc. Natl. Acad. Sci. U.S.A. **75**:117–121.
7. **De Larco, J. E., and G. J. Todaro.** 1978. Epithelioid and fibroblastic rat kidney cell clones: epidermal growth factor (EGF) receptors and the effect of mouse sarcoma virus transformation. J. Cell. Physiol. **94**:335–342.
8. **Deppert, W., K. Hanke, and R. Henning.** 1980. Simian virus 40 T-antigen-related cell surface antigen: serological demonstration on simian virus 40-transformed monolayer cells in situ. J. Virol. **35**:505–518.
9. **Deppert, W., and G. Walter.** 1982. Domains of simian virus 40 large T-antigen exposed on the cell surface. Virology **122**:56–70.
10. **Duff, R., F. Rapp, and J. S. Butel.** 1970. Transformation of hamster cells by variants of PARA-adenovirus 7 able to induce SV40 tumor antigen in the cytoplasm. Virology **42**:273–275.
11. **Fiers, W., R. Contreras, G. Haegeman, R. Rogiers, A. van de Voorde, H. van Heuverswyn, J. van Herreweghe, G. Volckaert, and M. Ysebaert.** 1978. Complete nucleotide sequence of SV40 DNA. Nature (London) **273**:113–120.
12. **Friedmann, T., A. Esty, P. LaPorte, and P. Deininger.** 1979. The nucleotide sequence and genome organization of the polyoma early region: extensive nucleotide and amino acid homology with SV40. Cell **17**:715–724.
13. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
14. **Harlow, E., L. V. Crawford, D. C. Pim, and N. M. Williamson.** 1981. Monoclonal antibodies specific for simian virus 40 tumor antigens. J. Virol. **39**:861–869.
15. **Ito, Y., J. R. Brocklehurst, and R. Dulbecco.** 1977. Virus-specific proteins in the plasma membrane of cells lytically infected or transformed by polyoma virus. Proc. Natl. Acad. Sci. U.S.A. **74**:4666–4670.
16. **Kalderon, D., W. D. Richardson, A. F. Markham, and A. E. Smith.** 1984. Sequence requirements for nuclear location of simian virus 40 large-T antigen. Nature (London) **311**:33–38.
17. **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.
18. **Land, H., L. F. Parada, and R. A. Weinberg.** 1983. Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature (London) **304**:596–602.
19. **Lane, D. P., and L. V. Crawford.** 1979. T antigen is bound to a host protein in SV40-transformed cells. Nature (London) **278**:261–263.
20. **Lanford, R. E., and J. S. Butel.** 1979. Antigenic relationship of SV40 early proteins to purified large T polypeptide. Virology **97**:295–306.
21. **Lanford, R. E., and J. S. Butel.** 1980. Inhibition of nuclear migration of wild-type SV40 tumor antigen by a transport-defective mutant of SV40-adenovirus 7 hybrid virus. Virology **105**:303–313.
22. **Lanford, R. E., and J. S. Butel.** 1980. Biochemical characterization of nuclear and cytoplasmic forms of SV40 tumor antigens encoded by parental and transport-defective mutant SV40-adenovirus 7 hybrid viruses. Virology **105**:314–327.
23. **Lanford, R. E., and J. S. Butel.** 1981. Effect of nuclear localization of large tumor antigen on growth potential of SV40-transformed cells. Virology **110**:147–158.
24. **Lanford, R. E., and J. S. Butel.** 1982. Intracellular transport of SV40 large tumor antigen: a mutation which abolishes migration to the nucleus does not prevent association with the cell surface. Virology **119**:169–184.
25. **Lanford, R. E., and J. S. Butel.** 1984. Construction and characterization of an SV40 mutant defective in nuclear transport of T antigen. Cell **37**:801–813.
26. **Linzer, D. I. H., and A. J. Levine.** 1979. Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. Cell **17**:43–52.
27. **Luthman, H., and G. Magnusson.** 1983. High efficiency polyoma DNA transfection of chloroquine treated cells. Nucleic Acids Res. **11**:1295–1308.
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., V. P. Setlow, C. A. F. Edwards, and D. Vembu.** 1979. The roles of the simian virus 40 tumor antigens in transformation of Chinese hamster lung cells. Cell **17**:635–643.
30. **McCormick, F., and E. Harlow.** 1980. Association of a murine 53,000-dalton phosphoprotein with simian virus 40 large-T antigen in transformed cells. J. Virol. **34**:213–224.
31. **Osborn, M., and K. Weber.** 1975. Simian virus 40 gene A function and maintenance of transformation. J. Virol. **15**:636–644.
32. **Rapp, F., S. Pauluzzi, and J. S. Butel.** 1969. Variation in properties of plaque progeny of PARA (defective simian papovavirus 40)-adenovirus 7. J. Virol. **4**:626–631.
33. **Rassoulzadegan, M., A. Cowie, A. Carr, N. Glaichenhaus, R. Kamen, and F. Cuzin.** 1982. The roles of individual polyoma virus early proteins in oncogenic transformation. Nature (London) **300**:713–718.
34. **Rassoulzadegan, M., B. Perbal, and F. Cuzin.** 1978. Growth control in simian virus 40-transformed rat cells: temperature-independent expression of the transformed phenotype in *ts*A transformants derived by agar selection. J. Virol. **28**:1–5.
35. **Rassoulzadegan, M., R. Seif, and F. Cuzin.** 1978. Conditions leading to the establishment of N (*a* gene dependent) and A (*a* gene independent) transformed states after polyoma infection of rat fibroblasts. J. Virol. **28**:421–426.
36. **Reddy, V. B., B. Thimmappaya, R. Dhar, K. N. Subramanian, B. S. Zain, J. Pan, P. K. Ghosh, M. L. Celma, and S. M. Weissman.** 1978. The genome of simian virus 40. Science **200**:494–502.
37. **Richardson, L. S., and J. S. Butel.** 1971. Properties of transformed hamster cells containing SV40 tumor antigen in the cytoplasm. Int. J. Cancer **7**:75–85.
38. **Risser, R., and R. Pollack.** 1974. A nonselective analysis of SV40 transformation of mouse 3T3 cells. Virology **59**:477–489.
39. **Ruley, H. E.** 1983. Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture. Nature (London) **304**:602–606.
40. **Santos, M., and J. S. Butel.** 1982. Association of SV40 large tumor antigen and cellular proteins on the surface of SV40-transformed mouse cells. Virology **120**:1–17.
40a. **Santos, M., and J. S. Butel.** 1985. Surface T-antigen expression in simian virus 40-transformed mouse cells: correlation with cell growth rate. Mol. Cell. Biol. **5**:1051–1057.
41. **Seif, R., and F. Cuzin.** 1977. Temperature-sensitive growth regulation in one type of transformed rat cells induced by the *ts*A mutant of polyoma virus. J. Virol. **24**:721–728.
42. **Seif, R., and R. G. Martin.** 1979. Simian virus 40 small t antigen is not required for the maintenance of transformation but may act as a promoter (cocarcinogen) during establishment of transformation in resting rat cells. J. Virol. **32**:979–988.
43. **Sleigh, M. J., W. C. Topp, R. Hanich, and J. F. Sambrook.** 1978. Mutants of SV40 with an altered small t protein are reduced in their ability to transform cells. Cell **14**:79–88.
44. **Soeda, E., J. R. Arrand, N. Smolar, J. E. Walsh, and B. E. Griffin.** 1980. Coding potential and regulatory signals of the polyoma virus genome. Nature (London) **283**:445–453.
45. **Soule, H. R., and J. S. Butel.** 1979. Subcellular localization of simian virus 40 large tumor antigen. J. Virol. **30**:523–532.
46. **Soule, H. R., R. E. Lanford, and J. S. Butel.** 1982. Detection of simian virus 40 surface-associated large tumor antigen by enzyme-catalyzed radioiodination. Int. J. Cancer **29**:337–344.
47. **Tegtmeyer, P.** 1972. Simian virus 40 deoxyribonucleic acid synthesis: the viral replicon. J. Virol. **10**:591–598.
48. **Tegtmeyer, P.** 1975. Function of simian virus 40 gene *A* in transforming infection. J. Virol. **15**:613–618.