JOURNAL OF VIROLOGY, Dec. 1979, p. 979-988
0022-538X/79/12-0979/10$02.00/0

Vol. 32, No. 3

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

ROLAND SEIF* AND ROBERT G. MARTIN

Laboratory of Molecular Biology, National Institute of Arthritis, Metabolism and Digestive Diseases, Bethesda, Maryland 20205

Received for publication 15 May 1979

Simian virus 40 deletion mutants affecting the 20,000-dalton (20K) t antigen and *tsA* mutants rendering the 90K T antigen temperature sensitive, as well as double mutants containing both mutations, induced host DNA synthesis in resting rat cells at the restrictive temperature. Nonetheless, the deletion mutants and double mutants did not induce transformation in resting cells even at the permissive temperature. On the other hand, the deletion mutants did induce full transformants when actively growing rat cells were infected; the transformants grew efficiently in agar and to high saturation densities on plastic. The double mutants did not induce T-antigen-independent (temperature-insensitive) transformants which were shown previously to arise preferentially from resting cells. Thus, small t antigen was dispensable for the maintenance of the transformed phenotype in T-antigen-dependent rat transformants (transformants derived from growing cells) and may play a role in the establishment of T-antigen-independent transformants. We attempt to establish a parallel between transformation induced by chemical carcinogens and simian virus 40-induced transformation.

Four assays have generally been used to test the transforming potential of simian virus 40 (SV40). They are (i) a survival assay, only applicable to primary cells or to cell lines that do not survive in sparse culture, in which infected cells are seeded on plastic at a very low cell density and the surviving cells that form flat or dense foci are selected; (ii) a low-density focus assay, in which the treated cells are plated at cell densities that allow active growth after a lag and dense foci are selected; (iii) a high-density focus assay, in which the treated cells are plated at cell densities approaching the saturation density and dense foci are selected; and (iv) a colony assay, in which the treated cells are suspended in agar and colonies are selected. Evidence in the literature, supported by the data presented in this report, suggests that transformants isolated by the different assays are not entirely comparable.

Deletion mutants in the early region (0.54 to 0.59 map units) of SV40 synthesize either an aberrant or no 20,000-dalton (20K) t antigen but a normal 90K T antigen (9, 35). All of these mutants transformed like the wild-type virus in the low-density focus assay (2, 22, 34) and in the survival assay (35) but were defective in their transforming capacity both in the agar assay (2, 11) and in the high-density focus assay (35). It has been reported that more than 90% of rat cell lines selected by the survival assay do not grow in suspension (35). Mutants of polyoma virus with lesions and characteristics analogous to the SV40 early deletion mutants have been isolated and characterized previously (1, 10, 12, 28, 29, 36).

Two transformed states have been inferred, based on studies using temperature-sensitive *A* gene mutants of SV40 (or *tsa* mutants of polyoma). When mutants containing a temperature-sensitive 90K T antigen were used to transform rapidly growing rat cells and growth was allowed to continue for several days postinfection, the resultant transformants were temperature sensitive (N transformants). Cells entering the resting state soon after infection gave rise to temperature-insensitive transformants (A transformants) (26, 32, 33).

In an attempt to determine the role of the 20K t antigen in the establishment and/or the maintenance of T-antigen-dependent (N) and T-antigen-independent (A) transformants, we used SV40 deletion mutants and double mutants containing both a deletion of the 20K t antigen and

a temperature-sensitive lesion in the 90K T antigen to induce cell transformation under different conditions. We report the following. (i) The ability or inability of the early deletion mutants to transform was not due to the transformation assay per se, but was related to the growth state of the normal cells under these different conditions; i.e., the deletion mutants were unable to transform resting cells but could transform growing cells. (ii) Transformants induced by SV40 deletion mutants did grow in agar and had high saturation densities. (iii) SV40 early deletions, *tsA* mutants, and deletion-*tsA* double mutants induced cellular DNA synthesis in resting nonpermissive rat cells at the restrictive temperature. And finally, (iv) the transformed rat cells generated by an SV40 double mutant were all temperature sensitive, suggesting that the inability of the small t antigen mutants to transform resting cells was related to their inability to induce T-antigen-independent transformants. Results suggesting that the deletion mutants were required for the establishment of transformation in resting CHL cells have been published elsewhere (22).

MATERIALS AND METHODS

Virus, cells, medium, and restriction enzyme. SV40 mutants *Δ890*, *Δ2005*, and *tsA209* have been described previously (4, 34, 35), as has the double mutant *Δ292tsA209* (V. P. Setlow, M. Persico-Di-Lauro, C. A. F. Edwards, and R. G. Martin, submitted for publication). This double mutant contains a *tsA* mutation and codes for an abnormal small t antigen because of a deletion of approximately 150 base pairs in the early region. No small t antigen has been detected upon lytic infection with this mutant. The *AluI* site within seven nucleotides of the donor splice site is missing. Virus was grown in CV1 cells at low initial multiplicities of infection (0.001 PFU/cell). The viral stocks were titrated by the plaque assay. The deletion mutants were routinely checked for purity by analysis of their restriction patterns. Normal rat FR3T3 cells have been described previously (32); rat wild-type SV40 transformants (WT-FR3T3-L5051 and H5011) and temperature-sensitive (A209-FR3T3-N51) and temperature-insensitive transformants (A209-FR3T3-A12, A209-FR3T3-A63) have also been described previously (33). Dulbecco modified Eagle medium containing 10% fetal calf serum (Flow Laboratories) was used for all cell lines. *Hinf* endonuclease was obtained from Bethesda Research Laboratories, Bethesda, Md. The same lot of serum was used in all experiments.

Crude lysates and partially purified virus. Infected CV1 cells were disrupted by freezing and thawing when the cytopathology was extensive, and the resultant mixture was used as a source of virus (crude lysate). Mock lysates of uninfected CV1 cells were also prepared; these lysates did not give a single plaque when assayed for SV40. Partially purified viral preparations were obtained by centrifuging the crude lysate at low speed (10,000 rpm, 30 min) and then mixing the supernatant with sufficient CsCl to bring the final density to 1.33g/cm^3. Samples were centrifuged overnight at 33,000 rpm and 15°C. The virus band at 1.33g/cm^3 was recovered and dialyzed overnight at room temperature against phosphate buffer (pH 7.4), and the viral suspension was titrated again.

Transformation assay. Rat 3T3 cells growing at low cell density (1 to 2% confluence) or at high cell density (30% confluence) or resting at confluence were infected with 5 to 20 PFU of virus (in lysate or buffer) per cell or mock infected with an uninfected CV1 lysate or buffer. The adsorption was carried out for 2 h at 33°C in a minimal volume (0.5 ml in a 25-cm^2 flask) while the cells were attached on the plastic surface. The infected cells were then suspended in agar (19) immediately (see Tables 1 and 3) or after 3 days (selection of the double transformants described in Table 6), or they were reseeded at different cell densities on plastic (see Tables 2 and 3). Alternatively, the infected cells were left on plastic for 1 week at 33°C and then suspended in agar or replated on plastic (see Table 4). After 5 to 6 weeks at 33°C, dense foci on plastic or colonies in agar were counted, isolated, and cloned as previously described (33). The mock-infected cells never gave rise to a single focus or colony. The medium containing 5 to 10% serum was changed or added every 5 to 7 days.

Saturation density and growth in agar. Saturation density and growth in agar were as previously described (33).

Virus rescue from transformed cells and preparation of viral DNA. Approximately 10^5 SV40-transformed cells were added to confluent CV1 cells in small flasks (approximately 10^6 cells per flask) and left for 2 weeks at 37°C (with regular medium change) until lysis of the culture was observed. The lysate was then used to infect CV1 cells, and the viral DNA was extracted by the Hirt procedure 36 h later at 37°C. DNA form I was isolated by using a cesium chloride-ethidium bromide gradient. The dye was removed by extraction with isopropanol saturated with CsCl, and the DNA was precipitated by ethanol and resuspended in a small volume of distilled water.

Cleavage by restriction endonuclease. The incubation mixture contained, in a total volume of 30 μl, 20 μl of DNA solution with approximately 1 μg of viral DNA, 3 μl of 10× enzyme buffer (60 mM Tris-hydrochloride, pH 7.5, 60 mM $MgCl_2$, 60 mM 2-mercaptoethanol, 200 mM NaCl), 6.5 μl of distilled water, and approximately 2 U of enzyme in 0.5 μl. The mixture was incubated for 1 h at 37°C, the reaction was stopped by adding 5 μl of 5% sodium dodecyl sulfate–25% glycerol in borate buffer, and 5 μl of a dye solution (2 mg of bromophenol blue per ml in borate buffer) was added.

Gel electrophoresis of DNA fragments. Separation of viral DNA fragments was carried out by electrophoresis through vertical slab gels (14 by 10 by 0.2 cm) containing 5% acrylamide and 0.17% bisacrylamide. A 40-μl sample was loaded into each slot, and electrophoresis was for 2 h at 150V in Tris-borate-EDTA buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH 8.2). DNA bands were stained with ethidium bromide and visualized by using short-wavelength UV light.

Induction of cellular DNA synthesis. Rat FR3T3 cells were grown to saturation density in 25-cm^2 flasks (10^6 cells per flask) and then incubated at 33°C for 2 days with medium containing 1% serum. The medium was removed and saved. These cultures were infected with 10 PFU of virus (wild type, *tsA209*, *Δ890*, or *Δ292tsA209*) per cell or mock infected with either phosphate buffer or uninfected CV1 lysate. The old medium was replaced, and the different cultures were pulse-labeled for 1 h with tritiated thymidine (1 μCi/ml of medium) at different times postinfection. After the labeling period, the cells were dissolved in 0.5 N NaOH. The DNA in a small sample was precipitated with 5% ice-cold trichloroacetic acid and retained on a glass fiber filter disk, and the radioactivity on the filter was determined.

Labeling, immunoprecipitation, and separation of viral proteins. Labeling, immunoprecipitation, and separation of the viral proteins from transformed cells were performed as previously described (9, 33).

RESULTS

20K t antigen was required for transformation of resting cells only. The inability of the deletion mutants to induce colonies in agar (2, 11) or dense foci by the high-density focus assay (35) appeared to us to have a common factor; namely, both conditions are generally restrictive for normal cell growth and favor entrance of normal cells into the resting state.

We decided to examine further the role of small t antigen in transformation of rat cells by using two early deletion mutants, *Δ890* (34) and *Δ2005* (35). *Δ890* was initially chosen because it was one of the most defective deletion mutants for transformation by the agar assay (2), although it gave rise to one transformed rat cell line selected by the survival assay that was capable of growth in methocel (35). It is a small deletion that can, however, be easily detected by restriction mapping. The 20K t antigen synthesized by this mutant is truncated (35). *Δ2005* was chosen because none of the rat cell lines induced by this mutant and selected by the survival assay grew in agar and because it is a much larger deletion than *Δ890* and synthesizes no 20K t antigen (35). Although it has been reported that *Δ890* is slightly less defective in permissive infection than *Δ2005*, the difference may reflect the fact that *Δ2005* contains a second deletion in the late region coding for VP_1 (see below). Since we found no difference in the transforming properties of these mutants, the results are presented jointly.

(i) When confluent, resting rat FR3T3 cells were infected with partially purified virus and subsequently suspended in agar, the induction of colony formation by the deletion mutant *Δ2005* was significantly reduced relative to wild-type virus (Table 1). This defect was less apparent with the unpurified virus preparation (crude lysate) (Table 1). (ii) When growing cells were infected and then immediately plated on plastic at different cell densities ranging from low (1% confluence) to high (100% confluence) to score for dense foci (see above), the defect of *Δ890* (Table 2) was observed only when partially purified virus was used and when the cells were plated at high densities after infection ($>2 \times 10^5$ cells per flask). No defect was observed if the cells were plated at low densities ($<2 \times 10^5$ cells per flask). Even when plated at high cell densities, *Δ890* was not defective when crude lysates were used instead of purified virus. (iii) When resting cells were infected with partially purified virus and then suspended in agar or plated on plastic at low, moderate, or high cell densities, the defect of *Δ2005* (Table 3) was apparent in agar and on plastic at high cell density, but absent on plastic at low cell density. (iv) When actively growing cells were infected and suspended in agar, no colonies were seen with the deletion mutant-infected cells (Table 3). (v) Finally, when growing cells were infected and active growth continued for 1 week before the cells were suspended in agar (Table 4), those cultures infected with *Δ2005* gave rise to colonies in agar

TABLE 1. *Transformation of resting cells by the deletion mutant Δ2005, using the agar assay: lysate containing virus versus partially purified virus*

Source of virus	Virus	No. of colonies in agar containing >500 cells
Crude lysate containing virus	*Δ2005*	11
	Wild type	35
Partially purified virus in buffer	*Δ2005*	0
	Wild type	27

TABLE 2. *Transformation by the focus assay: influence of cell density and source of virus on transformation by an SV40 early deletion mutant*

Source of virus	Virus	No. of dense foci per flask for initial seeding density of:					
		10^4[a]	2×10^4	4×10^4	2×10^5	5×10^5	10^6
Crude lysate	*Δ890*	2	6	15	40	73	>100
	Wild type	7	11	24	>75	>100	>200
Partially purified virus	*Δ890*	3	8	11	22	19	2
	Wild type	4	16	26	>80	>125	>200

[a] Number of cells per 25-cm^2 flask. The seeding densities were the densities immediately after infection at 10 to 20 PFU/cell.

TABLE 3. *Transformation by an SV40 early deletion mutant: influence of the cell state (growing or resting) on transformation*

Cell density per flask at the time of infection[a]	Culture conditions	Initial seeding density (no. of cells per flask) immediately postinfection[b]	No. of colonies or dense foci	
			Δ2005	Wild type
2×10^4 (growing)	Plastic	10^4	9	7
	Agar	10^4	0	5
10^6 (resting)	Plastic	10^4	4	6
		10^5	26	37
		8×10^5	2	>150
	Agar	10^5	0	42

[a] The state of the cells is indicated in parentheses.
[b] Immediately after infection at 5 to 10 PFU/cell, the cells were seeded on plastic or in agar at the concentrations given.

TABLE 4. *Transformation by an SV40 early deletion mutant: active growth during the first week after infection was sufficient to establish transformants capable of growth in agar*

Culture conditions after infection	No. of colonies or dense foci per flask	
	Δ2005	Wild type
Cells grown for 7 days and then suspended in agar[a]	14	23
Cells grown for 7 days and then replated on plastic[b]	22	35

[a] Cells growing at low density (10^4 cells per flask) were infected with 10 to 20 PFU of mutant or wild-type virus per ml, grown on plastic until confluence (10^6 cells per flask), and then suspended in agar (10^5 cells per flask).
[b] Cells growing at low cell density (10^4 cells per flask) were infected with mutant or wild-type virus, grown until confluence (10^6 cells per flask), replated on plastic at one-tenth the normal saturation density (10^5 cells per flask), and left until foci were detected.

almost as efficiently as the wild-type virus-infected cultures.

In an attempt to determine how the CV1 lysate was able to complement the defect of the mutants, resting cells were incubated for 2 h at 33°C with an uninfected CV1 lysate and examined after further incubation on plastic or in agar. Figure 1 shows the morphology of the lysate-treated culture as compared with the untreated cells at 1 week after treatment. It is clear that many of the cells have undergone at least one division in the lysate-treated culture in addition to the change in morphology. Among the cells treated with the uninfected lysate and suspended in agar, a small percentage underwent from two to five divisions when the cultures

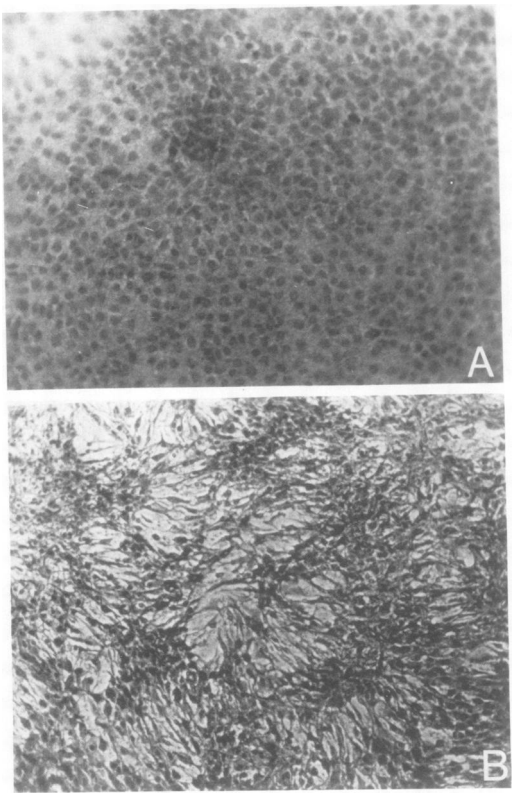

FIG. 1. *Effect of the uninfected CV1 lysate on resting rat cells at confluence. Normal rat FR3T3 cells at confluence were incubated for 2 h at 33°C with either phosphate buffer (A) or uninfected CV1 lysate (B), fed with medium containing 2% serum for 1 week, and then examined.*

were examined after 3 weeks (data not shown), whereas no microcolonies were found when untreated cells were used. No attempt has been made to purify the mitogenic agent(s) contained in the CV1 cell lysate. It is likely that many cellular proteins, including lysosomal proteases, were liberated when the cells were physically disrupted or underwent lysis due to viral replication. Thrombin and trypsin are capable of inducing cell division in normal resting cells (3, 30). Isolation of the virus in a CsCl gradient significantly reduced the amount of nonviral proteins.

Taken together, these results suggest that the small t antigen deletion mutants were unable to induce transformation in resting cells (at the saturation density on plastic or suspended in agar) but could transform growing cells, provided that growth was permitted to continue for a brief period postinfection. The deletion mutants also transformed resting cells induced to grow by the CV1 lysate. After 1 week of active

growth postinfection, the deletion mutants induced nearly as many colonies in agar as did wild-type virus (Table 4).

Deletion mutants induce full transformants. It can be deduced from Tables 1, 2, and 4 that cells transformed by the deletion mutants (i) grew in agar, (ii) grew over a monolayer of normal cells, and (iii) grew over each other (piling up to form dense foci). Nonetheless, we isolated several dense foci induced by treatment with the deletion mutants (using partially purified virus or virus from a lysate), cloned the cells, and analyzed their ability to form colonies in agar and to grow to high cell densities on plastic. All of the *Δ890-* and *Δ2005*-induced transformants grew efficiently in agar and to high saturation densities on plastic (Table 5). No difference was found by these criteria between the deletion mutant-transformed cell lines and three *tsA* mutant-transformed cell lines grown at 33°C, whether the latter cell line was a T-antigen-dependent (N) or T-antigen-independent (A) transformant isolated as a focus or a T-antigen-independent (A) transformant isolated as a colony in soft agar. The colony size in agar varied between 32 and 4,000 cells per colony for every cell line.

Virus was rescued from some of the transformed cell lines. The DNA was purified and digested with *Hinf*. In all cases the gel patterns of the restriction fragments were the same as those of the original transforming virus (Table 5 and Fig. 2). Viral proteins were extracted from some transformants and, none was found to contain a normal-size small t antigen (Table 2 and Fig. 3). We conclude that the deletion mutants can induce full transformants in rat cells.

Small t antigen is not required to induce DNA synthesis in resting cells. From the above results it appeared that the defect of the deletion mutants in cell transformation was at the establishment level (the early events during transformation) rather than at the maintenance level. Since the induction of host DNA synthesis is one of the earliest recognizable events during transforming infection, the capactiy of the various mutants to induce DNA synthesis was tested. Confluent, resting rat cells in low-serum medium were infected with wild-type virus, a temperature-sensitive mutant of the large T antigen (*tsA209*), a deletion mutant of the small t antigen (*Δ2005*), or a double mutant bearing both a deletion of the small t antigen and a temperature-sensitive mutation in the large T-antigen (*Δ292tsA209*). The cells were subsequently incubated at 33 or 40°C (nonpermissive temperature in the lytic cycle for the *tsA* mutant) (4), and DNA synthesis was monitored at various times during the first days postinfection (Fig. 4). Partially purified virus in phosphate buffer or virus in a crude lysate was used. Mock infections were carried out both with phosphate buffer and with an uninfected CV1 lysate. A clear difference was found between the two controls. The uninfected cell lysate induced some DNA synthesis at both temperatures. Therefore, part of the induction observed with the virus-

TABLE 5. *Growth properties of rat transformants induced by SV40 deletion mutants*

Cell line	Saturation density (× 10^5 cells/flask)	Growth in agar[a]	Rescued virus[b]	Viral protein(s)[c]
FR3T3	11	0	*wild type*	T, t
WT-FR3T3-L5051	>90	24		
A209-FR3T3-N51	>90	30		
A209-FR3T3-A12	>90	17		
A209-FR3T3-A63	>90	36		
890-FR3T3-1018	>95	26		
890-FR3T3-1019	>95	32		
890-FR3T3-1022	>95	18	*Δ890*	T, —
890-FR3T3-1024	>95	25		
890-FR3T3-1025	>95	24		
890-FR3T3-1033	>95	32	*Δ890*	T, —
890-FR3T3-1034	>95	21	*Δ890*	
890-FR3T3-1044	>95	25		
890-FR3T3-1045	>95	31		
890-FR3T3-1047	>95	14		
890-FR3T3-1048	>95	26	*Δ890*	T, —
890-FR3T3-1049	>95	28		
890-FR3T3-1051	>95	31		
890-FR3T3-1053	>95	36	*Δ890*	
890-FR3T3-1054	>95	40		
890-FR3T3-1058	>95	19		
890-FR3T3-1059	>95	35		
890-FR3T3-1064	>95	30		
890-FR3T3-1066	>95	19		
2005-FR3T3-2011	>95	21		
2005-FR3T3-2012	>95	17	*Δ2005*	T, —
2005-FR3T3-2014	>95	19	*Δ2005*	
2005-FR3T3-2015	>95	32	*Δ2005*	
2005-FR3T3-2017	>95	24		
2005-FR3T3-2018	>95	19		
2005-FR3T3-2022	>95	26		
2005-FR3T3-2024	>95	25	*Δ2005*	T, —
2005-FR3T3-2026	>95	18		
2005-FR3T3-2027	>95	28		
2005-FR3T3-2032	>95	32	*Δ2005*	T, —
2005-FR3T3-2033	>95	29		
2005-FR3T3-2041	>95	22		

[a] Colony formation efficiency as a percentage of the seeding density (10^5 cells per flask).

[b] The virus was rescued, and the viral DNA was digested by *Hinf* restriction enzyme (Fig. 2).

[c] The viral proteins were extracted, immunoprecipitated, and separated by gel electrophoresis (Fig. 3). —, No small t was detectable.

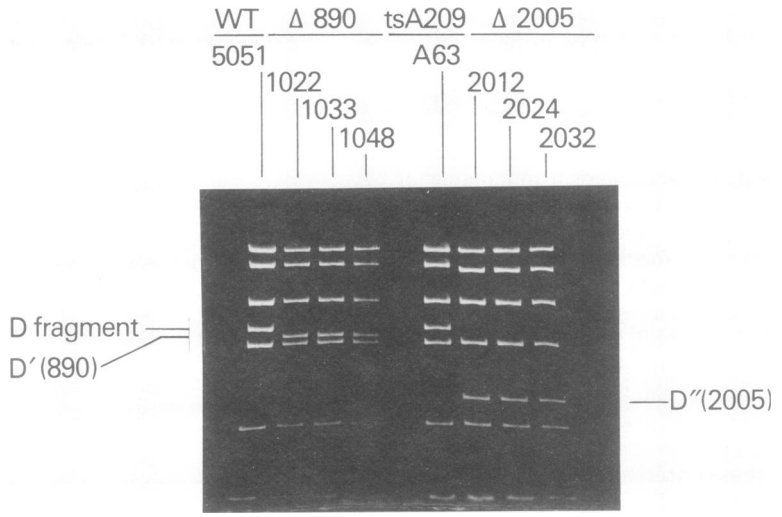

FIG. 2. *Hinf DNA restriction pattern of virus rescued from transformed cells. The virus rescued from some transformed cells (Table 5) was used to infect CV1 cells, and the viral DNA was isolated in an ethidium bromide-cesium chloride gradient. The DNA was digested by Hinf restriction enzyme, and the resulting fragments were separated by electrophoresis through a 5% acrylamide gel. The gel was impregnated with ethidium bromide, illuminated by a UV light, and photographed. Note that Δ2005 in addition to a shorter Hinf fragment D has a shorter Hinf fragment B. This second deletion is in the late portion of Hinf fragment B (EcoRII-fragment B), not in the portion covering the early region (EcoRII-fragment F). WT, Wild type.*

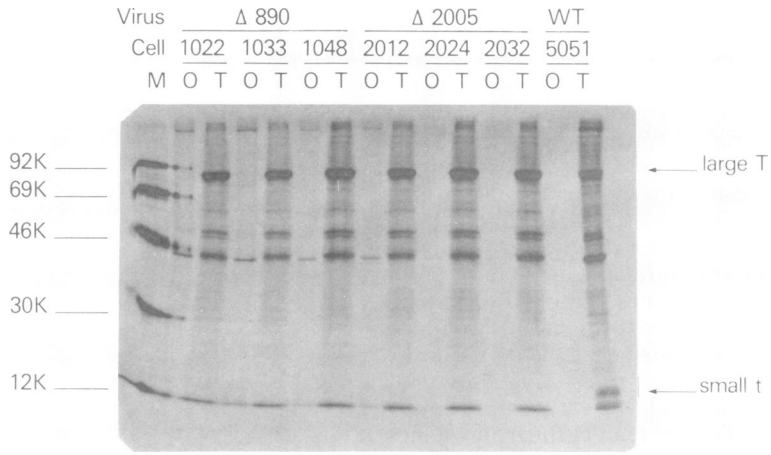

FIG. 3. *Viral proteins in transformed cells. [^{35}S]methionine-labeled nuclear extracts were prepared from some transformed cells (Table 5), and viral proteins were immunoprecipitated by using an anti-T serum (tracks labeled T) and protein A-Sepharose beads. A normal hamster serum (tracks labeled O) was used as a control. The precipitated proteins were separated by electrophoresis through a discontinuous polyacrylamide gel and revealed by fluorography. The track labeled M contained ^{14}C-labeled markers. WT, Wild type.*

containing lysate was not due to SV40. Nonetheless, even the partially purified preparation of the deletion mutant virus clearly induced cellular DNA synthesis to the same extend as the wild-type virus. Furthermore, the same induction of DNA synthesis was found with the *tsA* mutant and the deletion-*tsA* double mutant at 40°C. We conclude, therefore, that the 20K t antigen is not required for the induction of cellular DNA synthesis and that the block to transformation of resting cells by the small t antigen mutants is after the first round of virus-induced cellular DNA synthesis. We consider the role of the large T antigen in the induction of host DNA synthesis below.

Small t antigen is required to induce T-antigen-independent (A) transformants in resting rat cells. To determine the possible

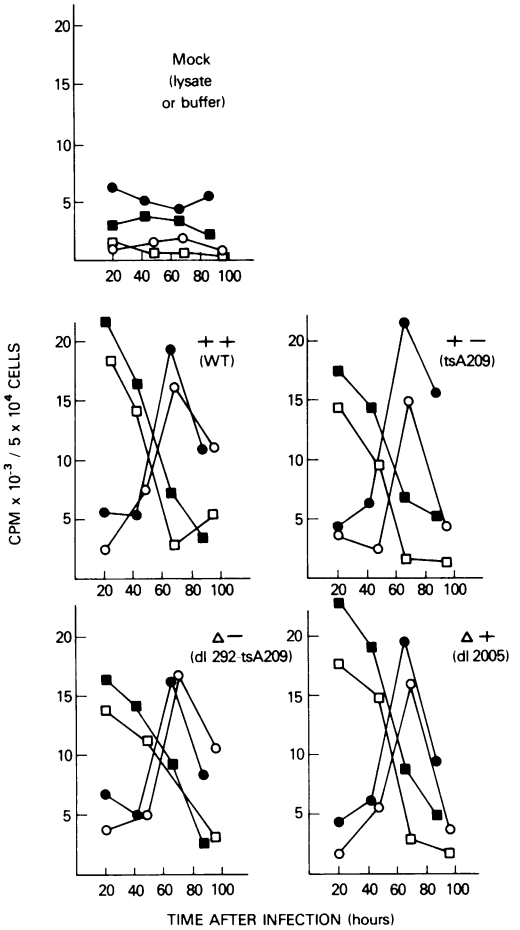

FIG. 4. *Induction of host DNA synthesis. Resting nonpermissive rat cells were infected with 10 PFU of virus (wild type [WT], tsA209, Δ890, or Δ292tsA209) per cell contained in a lysate (● and ■) or buffer (○ and □), or they were mock infected with an uninfected CV1 lysate or buffer. Cultures were incubated at either 33°C (● and ○) or 40°C (■ and □). DNA synthesis was monitored at the indicated times by pulse-labeling with [³H]thymidine, and the trichloroacetic acid-precipitable radioactivity was determined as indicated in the text.*

relationship among small t antigen, the resting state, and the occurrence of temperature-insensitive (A) transformants, we induced transformation with a double mutant under conditions where the *tsA* mutant alone gives rise to both temperature-sensitive (N) and temperature-insensitive (A) transformants (33). Rat cells growing at high densities on plastic (30% confluence) were infected with *tsA209* or *Δ292tsA209*, left for 3 days at 33°C, and then suspended in agar. After 6 weeks, approximately five times as many colonies were found in the cultures infected with *tsA209* as in the cultures infected with *Δ292tsA209*. Several colonies were isolated in agar, and their growth properties were studied at low and high temperatures. Table 6 shows that the cells infected with the *tsA* mutant gave rise, as expected, to both N and A transformants. Under these conditions none of the isolated double-mutant-induced transformants was temperature insensitive. We conclude that small t antigen may be required to induce A transformants in rat cells.

DISCUSSION

Full and intermediate transformants. Sleigh et al. (35) found that most foci (>90%) selected by the survival assay after infection of rat cells with early SV40 deletion mutants failed to grow in suspension and had low saturation densities. In an attempt to explain these results and the inability of the deletion mutants to induce colonies in agar (2, 11) or dense foci by the high-density focus assay (35), they suggested that the small t antigen was necessary for full transformation (27). Our results are not in agreement with the conclusion reached by Sleigh et al. (35); i.e., all of our deletion-induced dense foci selected by the low-density focus assay were full transformants, they grew in agar, and they had high saturation densities. Full transformants have also been observed with an analogous deletion of the human papovavirus BKV. Both BKV prototype and BKV strain Dunlop have a genome with a DNA sequence very similar tothat of wild-type SV40 (I. Seif, G. Khoury, and R. Dhar, Cell, in press). BKV strain MM, isolated from a brain tumor, has a naturally occurring deletion in the early region coding exclusively for small t antigen (31, 39); it is, therefore, comparable to an SV40 small t antigen deletion mutant. This virus (BKV strain MM) can induce cerebral tumors in hamsters (8) and transform both primary rabbit kidney cells (23) and primary mouse kidney cells (7) by the dense focus assay, and the selected transformants grow efficiently in agar (23) and are tumorigenic in nude mice (7).

Small t antigen and transformation. Our results confirm, as previously reported (2, 11, 35), that the mutants affecting the small t antigen are incapable of inducing the formation of colonies in agar or dense foci on a monolayer but are fully capable of transforming cells in the low-density focus assay on plastic (2, 34). In addition, our results suggest that the determining factor for detecting the transformation capacity or incapacity of the small t antigen mutants was unrelated to the transformation assay per se, but was dependent on the growth state of the in-

TABLE 6. *Growth properties of rat transformants induced by an SV40 deletion-tsA double mutant*

Cell line	Saturation density (×10⁵ cells/flask) at:		Growth in agar at:[a]		Transformation type[b]
	33°C	40.5°C	33°C	40.5°C	
FR3T3			0	0	
WT-FR3T3-H5011			33	29	
Δ292tsA209-FR3T3-H411			36	<0.01	N
Δ292tsA209-FR3T3-H414			34	<0.01	N
Δ292tsA209-FR3T3-H415			30	<0.01	N
Δ292tsA209-FR3T3-H417	>90	10	24	<0.01	N
Δ292tsA209-FR3T3-H419			52	<0.01	N
Δ292tsA209-FR3T3-H421			60	<0.01	N
Δ292tsA209-FR3T3-H423			40	<0.01	N
Δ292tsA209-FR3T3-H426			28	<0.01	N
Δ292tsA209-FR3T3-H442			44	<0.01	N
Δ292tsA209-FR3T3-H443	>90	10	36	<0.01	N
Δ292tsA209-FR3T3-H446	>95	10	23	<0.01	N
Δ292tsA209-FR3T3-H450			33	<0.01	N
Δ292tsA209-FR3T3-H451			33	<0.01	N
Δ292tsA209-FR3T3-H462	>90	11	37	<0.01	N
Δ292tsA209-FR3T3-H465			32	<0.01	N
Δ292tsA209-FR3T3-H466			42	<0.01	N
Δ292tsA209-FR3T3-H463			38	<0.01	N
Δ292tsA209-FR3T3-H427			70	<0.01	N
Δ292tsA209-FR3T3-H455			46	<0.01	N
Δ292tsA209-FR3T3-H481			41	<0.01	N
Δ292tsA209-FR3T3-H482			44	<0.01	N
Δ292tsA209-FR3T3-H487			26	<0.01	N
Δ292tsA209-FR3T3-H488			54	<0.01	N
Δ292tsA209-FR3T3-H486			47	<0.01	N
Δ292tsA209-FR3T3-H484	>95	10	41	<0.01	N
Δ292tsA209-FR3T3-H495			47	<0.01	N
12 tsA209-FR3T3			26–65	25–46	12A
3 tsA209-FR3T3	>90	10	18–54	<0.01	3N

[a] See Table 5, footnote *a*.

[b] N, Temperature sensitive; A, temperature insensitive.

fected cells (growing or resting) for a short period after infection. A similar conclusion has been reached by using hamster cells; however, the influence of the cell state was limited to the moment of infection (22). We conclude that once the transformed state had been established among growing rat cells, small t antigen was not required for its maintenance.

The discrepancy between our results and those of Sleigh et al. (35) could be understood by assuming that the difference in phenotype is related to the cells and the selection procedure used. One possible explanation is that under restrictive conditions for normal cells (e.g., the survival assay) the deletion mutants can confer to primary cells the ability to clone but not to become full transformants. However, under growing conditions the small t antigen mutants can give rise to full transformants. If this explanation is correct, the small t antigen would be dispensable for both immortalization and full transformation but transiently required to establish the transformed state in resting cells. Alternatively, the phenotypic difference observed might result from differences in the growth factors contained in the serum.

Induction of host DNA synthesis. The small t antigen was not required for inducing the first round of cellular DNA synthesis after infection by SV40. Since the *tsA* mutant (4) and the double mutant were found to induce a round of DNA synthesis at 40°C, we favor the hypothesis that the first induction of cellular DNA synthesis at 40°C was due to a residual activity of the large T antigen. At 42.5°C, the stimulation of host DNA synthesis by a *tsA* mutant was more deficient than at 40°C (6). A DNA fragment from the early region of SV40 containing coding sequences for small t antigen and part of large T antigen was unable to induce DNA synthesis when injected into resting cells (24), whereas a DNA fragment containing additional coding sequences toward the C terminal end of large T antigen did induce DNA synthesis (24). Finally, injection of pure large T antigen into resting cells induced DNA synthesis (37). Taken to-

gether, these results suggest that small t antigen is neither required for nor capable of inducing the first round of DNA synthesis in resting cells. It is possible that small t antigen is required to stimulate subsequent cell divisions.

Similar results have been reported when *tsa* and *hrt* mutants of polyoma virus were used to infect resting rat cells (29).

Transformation of resting cells. Resting rat cells gave rise to SV40 T-antigen-independent (A) transformants (33). We show here, using an SV40 double mutant, that the T-antigen-independent transformed state did not occur in rat cells without a wild-type small t antigen.

A similar picture emerges from the study of transformation of resting rat cells using polyoma virus. *tsa* mutants gave rise to T-antigen-independent transformants (26, 32), and *hrt* mutants failed to induce transformation (1, 10, 12).

We therefore conclude that both large T antigen and small t antigen are required in resting rat cells to establish a transformed state that is large T independent for maintenance. The experimental results showing an inability of SV40 *tsA* mutants (and polyoma virus *tsa* mutants) to transform at high temperature (12, 13, 21, 26, 33) suggest that the defect in transformation has to be at a level other than the induction of the first S phase. It is likely that large T antigen plays a role in the integration of viral DNA. The results on transformation of resting rat cells by the small t antigen deletion mutants (that code for an active large T antigen) suggest that a "barrier" to transformation of resting cells still exists beyond the first induced S phase and that this barrier can be "removed" by small t antigen.

Possible promoter-like activity of small t antigen. An explanation to account for these results is possible if one assumes (33) that T-antigen-independent (A) transformants arise from resting cells as a result of a mutation in a host gene involved in growth control. In a diploid cell, considering that the mutation is recessive, it is necessary to introduce an alteration in both alleles of the regulatory gene(s) or prevent the expression of the nonmutated allele (by loss, exclusion, or replacement) to have expression of the transformed phenotype. We suggest that small t antigen has this type of activity.

Kinsella and Radman (15) have proposed recently that promotion in chemical carcinogenesis is achieved by induction of aberrant chromosomal segregation, arrangement, distribution, or exchange, which allows the expression of recessive mutations in cells treated by initiators. In agreement with their hypothesis, griseofulvin, a chemical promoter, induces tetraploidy followed by rapid chromosomal loss (16) and then a rise to tetraploidy again. (The wild-type allele might be lost during the period of chromosome loss.)

Both polyoma virus (29) and SV40 (14, 17, 18) wild types induce permanent tetraploidy in rat, mouse, and hamster cells, whereas polyoma virus *hrt* mutants fail to do so (29). Polyoma virus and SV40 FR3T3 A transformants are tetraploid, but not all tetraploid cells induced by polyoma virus are transformed (29). Furthermore, both polyoma virus and SV40 small t antigens disorganize actin microfilaments, whereas large T antigens do not (29; A. Graessman, personal communication).

Griseofulvin induces chromosomal aberrations by disrupting microtubules (38) and dissolving the mitotic spindle (20). Actin microfilaments and microtubules are part of the mitotic spindle (40). It is therefore tempting to propose (Fig. 5) that small t antigen acts at the mitotic level in resting cells induced to divide by large T antigen and has a griseofulvin-like activity that may provide the needed promotion for "initiated" A-transformants. Alternatively, small t antigen, like phorbol esters, may promote transformation by inducing abnormal chromosome exchange (15) without altering the chromosome number.

Transformants obtained from growing cells. We previously proposed (33) (i) that integration of viral DNA within a growth control gene is an unfrequent event in growing cells, which becomes more frequent in cells submitted to restrictive conditions, and (ii) that T-antigen-dependent (N) transformants arising from growing cells have genetically intact growth control

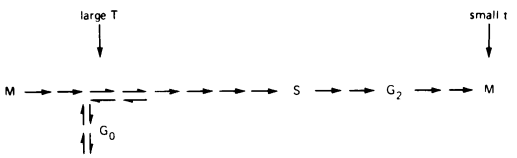

FIG. 5. *Possible roles of SV40 large T antigen and small t antigen in cell transformation. Large T antigen acting at the restriction point (25) triggers a cascade of events leading to S phase. This same activity maintains the ability of N transformants to grow under suboptimal conditions. Large T antigen is also required before or during the induced S phase to integrate the viral DNA into one allele of a cellular gene involved in growth control (initiation of A-transformation). However, the transformed phenotype cannot be established unless the nonmutated allele is eliminated. Small t antigen is proposed to alter the mitotic spindle, inducing aberrations in chromosomal segregation or exchange, which may result in the elimination of the nonmutant allele. A-transformation does not need viral proteins for maintenance. G_0 is represented as a multiple-step resting state in the early part of G_1.*

genes inactivated by large T antigen at the protein level. This dominant interaction does not require a promotional event, which may explain why small t antigen is dispensable for the transformation of growing cells.

LITERATURE CITED

1. **Benjamin, T. L.** 1970. Host range mutants of polyoma virus. Proc. Natl. Acad. Sci. U.S.A. **67**:394-399.
2. **Bouck, N., N. Beales, T. Shenk, P. Berg, and G. DiMayorca.** 1978. New region of the simian virus 40 genome required for efficient viral transformation. Proc. Natl. Acad. Sci. U.S.A. **75**:2473-2477.
3. **Carney, D. H., and D. D. Cunningham.** 1978. Cell surface action of thrombin is sufficient to initiate division of chick cells. Cell **14**:811-823.
4. **Chou, J. Y., J. Avila, and R. G. Martin.** 1974. Virial DNA synthesis in cells infected by temperature-sensitive mutants of simian virus 40. J. Virol. **14**:116-124.
5. **Chou, J. Y., and R. G. Martin.** 1974. Complementation analysis of simian virus 40 mutants. J. Virol. **13**:1101-1109.
6. **Chou, J. Y., and R. G. Martin.** 1975. DNA infectivity and the induction of host DNA synthesis with temperature-sensitive mutants of simian virus 40. J. Virol. **15**: 145-150.
7. **Costa, J., P. M. Holley, F. Legallais, C. Yee, N. Young, and A. S. Rabson.** 1977. Oncogenicity of a nude mouse cell line transformed by a human papovovirus. J. Natl. Cancer Inst. **58**:1147-1149.
8. **Costa, J., C. Yee, T. S. Pralka, and A. S. Rabson.** 1976. Hamster ependymomas produced by intercerebral inoculation of a human papova virus (MMV). J. Natl. Cancer Inst. **56**:863-864.
9. **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.
10. **Eckhart, W.** 1977. Complementation between temperature-sensitive (ts) and host range nontransforming (hr-t) mutants of polyoma virus. Virology **77**:589-597.
11. **Feunteun, J., M. Kress, M. Gardes, and R. Monier.** 1978. Viable deletion mutants in the simian virus 40 early region. Proc. Natl. Acad. Sci. U.S.A. **75**:4455-4459.
12. **Fluck, M., R. Staneloni, and T. L. Benjamin.** 1977. Hr-t and ts-a: two early gene functions of polyoma virus. Virology **77**:610-624.
13. **Fried, M.** 1965. Cell transforming ability of a temperature sensitive mutant of polyoma virus. Proc. Natl. Acad. Sci. U.S.A. **53**:486-491.
14. **Horan, P., J. Jett, A. Romero, and J. Lehman.** 1974. Flow microfluorometry analysis of DNA content in Chinese hamster cells following infection with simian virus 40. Int. J. Cancer **14**:514-521.
15. **Kinsella, A. R., and M. Radman.** 1978. Tumor promoter induces sister chromotid exchanges: relevance to mechanism of carcinogenesis. Proc. Natl. Acad. Sci. U.S.A. **75**:6149-6153.
16. **Larizza, L., G. Simoni, F. Tredici, and L. DeCarli.** 1974. Criseofulvin: a potential agent of chromosomal segregation in cultured cells. Mutat. Res. **25**:123-130.
17. **Lehman, J., and V. Defendi.** 1970. Changes in deoxyribonucleic acid synthesis regulation in Chinese hamster cells infected with simian virus 40. J. Virol. **6**:738-749.
18. **Lehman, J., J. Mavel, and V. Defendi.** 1971. Regulation of DNA synthesis in microphages infected with simian virus 40. Exp. Cell Res. **67**:230-233.
19. **MacPherson, I., and L. Montagnier.** 1964. Agar suspension cultures for the selective assay of cells transformed by polyoma virus. Virology **23**:291-294.
20. **Malawista, S. E., H. Sato, and W. A. Creasey.** 1976. Dissociation of the mitotic spindle in oocytes exposed to griseofulvin. Exp. Cell Res. **99**:193-196.
21. **Martin, R. G., and J. Y. Chou.** 1975. Simian virus 40 function required for the establishment and maintenance of malignant transformation. J. Virol. **15**:599-612.
22. **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.
23. **Mason, D. H., and K. K. Takemoto.** 1977. Transformation of rabbit kidney cells by BKV (MM) human papovavirus. Int. J. Cancer **19**:391-395.
24. **Mueller, C., A. Graessmann, and M. Graessman.** 1978. Mapping of early SV40-specific functions by microinjection of different early viral DNA fragments. Cell **15**: 579-585.
25. **Pardee, A. B.** 1974. A restriction point for control of normal animal cell proliferation. Proc. Natl. Acad. Sci. U.S.A. **71**:1286-1290.
26. **Rassoulzadegan, M., R. Seif, and F. Cuzin.** 1978. Conditions leading to the establishment of the N (*a* gene dependent) and A (*a* gene independent) transformed states after polyoma virus infection of rat fibroblasts. J. Virol. **28**:421-426.
27. **Risser, R., and R. Pollack.** 1974. A non-selective analysis of SV40 transformation of mouse 3T3 cells. Virology **59**:477-489.
28. **Schauffhausen, B. S., J. E. Silver, and T. L. Benjamin.** 1978. Tumor antigen(s) in cells productively infected by wild-type polyoma virus and mutant NG-18. Proc. Natl. Acad. Sci. U.S.A. **75**:79-83.
29. **Schlegel, R., and T. L. Benjamin.** 1978. Cellular alterations dependent upon the polyoma virus hr-t function: separation of mitogenic from transforming capacities. Cell **14**:587-599.
30. **Sefton, B. M., and H. Rubin.** 1970. Release from density dependent inhibition by proteolytic enzymes. Nature (London) **227**:843-845.
31. **Seif, I., G. Khoury, and R. Dhar.** 1979. BKV splice sequences based on analysis of preferred donor and acceptor sites. Nucleic Acids Res. **6**:3387-3398.
32. **Seif, R., and F. Cuzin.** 1977. Temperature-sensitive growth regulation in one type of transformed rat cells induced by the *tsa* mutant of polyoma virus. J. Virol. **24**:721-728.
33. **Seif, R., and R. G. Martin.** 1979. Growth state of the cell early after infection with simian virus 40 determines whether the maintenance of transformation will be *A*-gene dependent or independent. J. Virol. **31**:350-359.
34. **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.
35. **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.
36. **Staneloni, R., M. Fluck, and T. L. Benjamin.** 1977. Host range selection of transformation-defective hr-t mutants of polyoma virus. Virology **77**:598-609.
37. **Tjian, R., G. Fey, and A. Graessmann.** 1978. Biological activity of purified simian virus 40 T antigen proteins. Proc. Natl. Acad. Sci. U.S.A. **75**:1279-1283.
38. **Weber, K., J. Wehland, and W. Herzog.** 1976. Griseofulvin interacts with microtubules both in vivo and in vitro. J. Mol. Biol. **102**:817-829.
39. **Yang, R. C. A., and R. Wu.** 1979. BK virus DNA sequence: extent of homology with simian virus 40 DNA. Proc. Natl. Acad. Sci. U.S.A. **76**:1179-1183.
40. **Zacheus Cande, W., E. Lazarides, and J. R. McIntosh.** 1977. A comparison of the distribution of actin and tubulin in the mammalian mitotic spindle as seen by indirect immunofluorescence. J. Cell Biol. **72**:552-567.