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Large T Antigens of Simian Virus 40 and Polyomavirus Efficiently Establish Primary Fibroblasts

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Recombinant retroviruses that transduce the simian virus 40 (SV40) large T antigen or the polyomavirus large T antigen as well as encoding resistance to antibiotic G418 were used to investigate whether these genes alone were sufficient for immortalization of primary cells. The results provided definitive evidence that either viral gene can efficiently establish primary fibroblasts. The capability of the SV40 large T antigen to establish primary fibroblasts was undiminished by a mutation that alters its binding to sequences within the origin of replication. Surprisingly, most of the primary cells established by the expression of the SV40 large T antigen did not have a transformed phenotype. This suggests that transformation by SV40 is not simply due to a high level of expression of the SV40 large T antigen and stabilization of cellular p53.

Although recent work has shown that the polyomavirus (Py) large T antigen, the simian virus 40 (SV40) large T antigen, the cellular p53 protein, the adenovirus EIa protein, and the *myc* protein are all capable of establishing primary cells in vitro (4, 8, 12, 20, 22, 24–26), the efficiency of this oncogene-cell interaction has not been addressed. For example, establishment might require cellular mutations in addition to expression of the oncogene. We have addressed both questions by using recombinant retroviruses that encode the SV40 large T, Py large T or Py middle T antigen as well as resistance to antibiotic G418. In addition, we have tested a retrovirus which transduces a mutant SV40 large T antigen (SV-U19) which does not bind to the SV40 origin of replication to investigate whether this lack of binding affects its ability to establish primary cells (21).

Either secondary or tertiary rat embryo fibroblasts (REFs) prepared from 12- to 14-day-old Fischer rat embryos were infected with recombinant retroviruses that transduced the SV40 large T, SV-U19 large T, Py large T, or Py middle T antigen or with control virus [backbone only, Zip Neo SV(X)I] (2). The construction and characterization of the DNA recombinants as well as the subsequent isolation of the Ψ_2-derived retrovirus producer cell lines have been described elsewhere (11). These producer cell lines yield helper-free retrovirus packaged in an ecotropic Moloney murine leukemia virus particle. Since the retroviruses were constructed by using the Zip Neo SV(X)I vector system, the resulting provirus encoded both resistance to antibiotic G418 and the inserted gene (2, 3). At 48 h after infection, the cultures were passaged and subjected to selection with 0.3 mg of G418 per ml. After 14 days, the number of G418-resistant (G418^r) colonies obtained was counted, and the virus titer was determined. Although the data shown in Table 1 indicate that the viral titers on REFs were variable, they clearly reflect the titers of the same virus stock on either established mouse (NIH 3T3, data not shown) or established rat (F111, Table 1) fibroblast cell lines, even though they were decreased by about 10- to 20-fold. Since all the viral titers were reduced by about the same magnitude, the loss in viral titer probably reflects a lower efficiency of infection of primary fibroblasts than of established fibroblast cell lines.

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For each recombinant retrovirus, a large number of G418^r colonies were selected and isolated in 24-well microtiter dishes. Clones that grew were expanded into cell lines and passaged for 10 generations. Of the 15 G418^r colonies derived from infection with the control virus [Zip Neo SV(X)I], which only encodes resistance to G418, 10 grew after plating in the microtiter dishes. All of these cell lines entered a crisis period during passage 3, 4, or 5. Before entering crisis they grew faster than any of the other REF cell lines. Near the end of crisis (2 to 3 weeks), during which the medium was changed every 4 days, a few cells began to grow in two of the cultures. We concluded that these cell lines were established by some spontaneous event which permitted passage through crisis.

An equivalent set of G418^r colonies was selected after infection with the retrovirus encoding the Py middle T antigen. All of these G418^r colonies had the Py middle T antigen-transformed morphology. Very few of the G418^r colonies isolated from this infection grew in the microtiter dishes. Only 2 of 13 colonies in the first experiment and only 1 of 9 colonies in the second experiment began to grow after plating in the microtiter dishes. These cultures all entered a crisis period at a passage even earlier than those derived from the infection with the control virus. After a few weeks, during which there was no growth, one culture yielded a cell line that rapidly proliferated. This is the fastest growing cell line (doubling time, ~16 h) that we have so far isolated from REFs.

In contrast, the results obtained with the retroviruses encoding the Py and SV40 large T antigens were vastly different. We established 20 cell lines from a total of 33 G418^r colonies isolated after infection with the wild-type SV40 large T antigen. None of these went through a crisis period, and all of the colonies that grew in the microtiter dishes were established as cell lines. These results indicated that at least 60% of the initial G418^r colonies produced established cell lines (Table 2). Similarly, when colonies derived after infection with the mutant SV-U19 large T antigen were isolated and passaged, all of them (six of six, Table 2) were established without crisis. The results with the Py large T antigen were similar, but a few differences were observed. Unlike colonies isolated from infection with the SV40 large T antigen, only about 70% of the colonies that initiated growth

TABLE 1. G418ʳ titers of the SV40 large T, Py large T, and Py middle T antigen recombinant producer cell lines[a]

Virus	Insert	Protein transduced	Titer (G418ʳ CFU/ml) on:	
			Rat F111 fibroblasts	REFs
Zip Neo SV(X)I	Backbone only		3×10^5	3×10^4
LT-4 and LT-5	Py large T antigen	Py large T antigen	6×10^4	2.8×10^3
MT-5	Py middle T antigen	Py middle T antigen	6×10^3	7×10^2
SV40-6	SV40 early region	SV40 large T antigen	2×10^4	3×10^3
SV-U19-5	SV-U19 early region	SV-U19 large T antigen	ND[b]	5×10^4

[a] Cultures of REFs prepared (23) from 12- to 14-day-old Fischer rat embryos were generously provided by H. Land. Approximately 10^6 cells seeded in 10-cm dishes in Dulbecco modified Eagle medium supplemented with 10% (vol/vol) fetal calf serum, penicillin, and streptomycin (normal medium) were infected with the recombinant retroviruses. Infections were carried out in 2 ml of normal medium containing 0.2 ml of virus stock and 8 μg of Polybrene (Aldrich Chemical Co.) per ml for 2 h, after which 8 ml of normal medium was added. After incubation of the cultures for 48 h at 37°C, the cultures were split, and resistant cells were selected in normal medium containing 0.3 mg of G418 (GIBCO Laboratories) per ml. (3). Cultures were refed every 4 days, and the number of resistant colonies was counted 14 days after selection.

[b] ND, Not done.

(grew in microtiter wells) established cell lines without crisis, and one did so after crisis. Thus, about 50% of the G418ʳ colonies gave rise to cell lines that were established without crisis. Generally, most of these cell lines grow more slowly than those established with the wild-type or mutant SV40 large T antigen (unpublished observations). None of the Py large-T-antigen- or SV40 large-T-antigen-established cell lines had a morphology which was vastly different from that of the original REFs. The fact that at least 50% of the G418ʳ colonies obtained after infection with retroviruses encoding either the Py or the SV40 large T antigen were capable of establishing primary fibroblasts in vitro without crisis strongly suggests that these oncogenes alone are sufficient for establishment. Moreover, the fact that retroviruses encoding the mutant SV-U19 large T antigen established cell lines at least as efficiently as did retroviruses encoding the wild-type large T antigen demonstrates that large T antigen binding to the origin sequences is not required for efficient establishment of primary fibroblasts.

A number of the above-mentioned cell lines were analyzed to determine whether they expressed the appropriate protein. Cells were metabolically labeled with [^{35}S]methionine, and equal amounts of acid-precipitable radioactivity were immunoprecipitated with either monoclonal antibodies specific for the SV40 large T antigen or anti-Py T-antigen serum. Analysis of the expression of the SV40 large T antigen in five lines immortalized by the wild-type gene (RSV4062, RSV4068, RSV40611, R2SV4061, and R2SV40613) is shown in Fig. 1; results for the mutant SV-U19 gene (RSVU1951, RSVU1952, RSVU1954, RSVU1955, and RSVU1956) are shown in Fig. 2. Extracts prepared from COS M6 cells, a subclone of COS cells isolated by Horowitz et al. (7), RSV2a13, a REF cell line derived from infection with the control retrovirus Zip Neo SV(X)I, and R3LT44, a cell line established by the Py large T antigen, were included as positive and negative controls. In the analysis of cell lines established by the wild-type large T antigen gene, two different experiments are shown because, under the experimental conditions used for Fig. 1B, the large T antigen-p53 complex was not stable, whereas under the conditions used for Fig. 1A, the large T antigen-p53 complex was stable, but the background was higher. Together, the results shown in Fig. 1A and B and Fig. 2 clearly demonstrate that cell lines established by either the SV40 or the SV-U19 large T antigen recombinant retroviruses expressed high levels of large T antigen. This level of protein was not significantly different

TABLE 2. Immortalization of secondary REFs[a]

Virus	Expt	No. of G418ʳ colonies picked	No. that initiated growth	No. of cell lines established	No. established after crisis	No. established without crisis	Frequency of immortalization without crisis (%)
Zip Neo SV(X)I	1	15	10	2	2	0	0
LT-5	1	16	8	6	1	5[b]	31
	2	15	12	10	0	10	67
LT-4	2	16	10	7	0	7	44
SV40-6	1	15	8	8	0	8	53
	2	18	12	12	0	12	67
MT-5	1	13	2	0	0	0	0
	2	9	1	1	1	0	0
SV-U19-5	3[c]	6	6	6	0	6	100

[a] Approximately 14 days after selection with 0.3 mg of G418 per ml, a number of G418ʳ colonies were selected and isolated on 24-well microtiter dishes in normal medium by using cloning cylinders. Clones that grew were expanded into cell lines and passaged for 10 generations. All cell lines were refed every 4 days with fresh normal medium, even when they had entered crisis and stopped growing.

[b] Although one of these did not enter crisis, it grew much more slowly than the others.

[c] In the first two experiments, secondary REFs were used, whereas in the third experiment, tertiary REFs were used.

from that expressed in COS M6 cells. In addition, these retrovirus-established cell lines also contained elevated levels of cellular p53. The cellular p53 protein has been shown to increase in SV40-transformed or lytically infected cells, owing to its stabilization by association with the large T antigen (17–19). Analysis of the expression of the Py large T antigen in eight cell lines established by the Py large T antigen recombinant retrovirus is shown in Fig. 3. Once

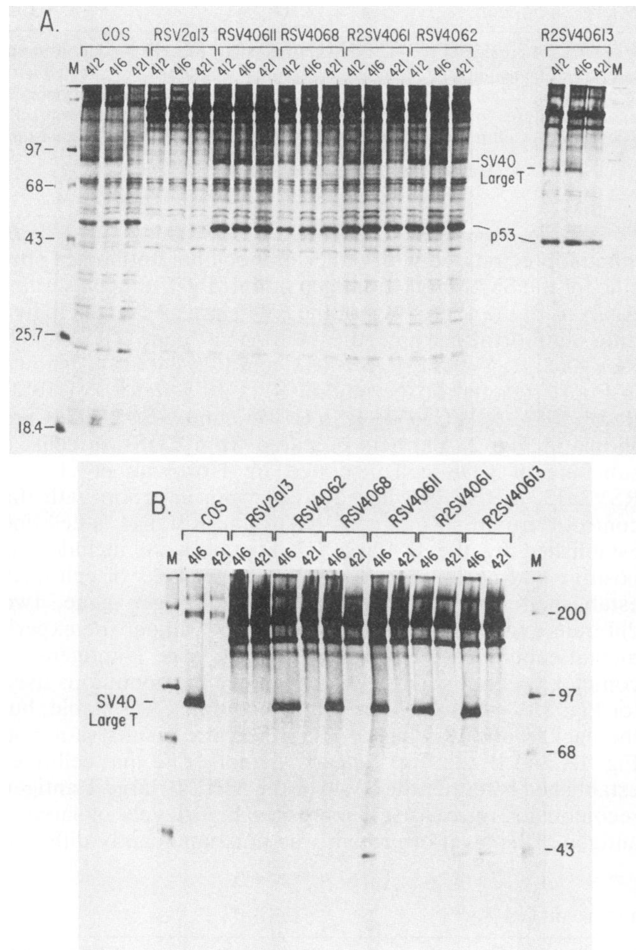

FIG. 1. Immunoprecipitation analysis of SV40 large-T-antigen-established cell lines. Cell lines established by either the wild-type large T antigen (RSV4062, RSV4068, RSV40611, R2SV4061, and R2SV40613) or the control retrovirus (RSV2a13) as well as COS M6 cells were labeled with 150 to 200 μCi of [^{35}S]methionine for 3 h. Proteins were extracted with either 100 mM Tris hydrochloride (pH 8.0–0.1 M NaCl–0.5% Nonidet P-40–1.6 TIU of aprotinin per ml (panel A) or RIPA (10 mM Tris hydrochloride [pH 7.2], 0.15 M NaCl, 1% Triton X-100, 1% sodium dodecyl sulfate, 1% sodium deoxycholate, 1.6 TIU of aprotinin per ml) lysis buffer (panel B). To permit comparisons of the relative levels of T-antigen synthesis, equal amounts of acid-precipitable radioactivity (4.4 × 10^6 cpm [panel A] and 2.5 × 10^6 cpm [panel B]) were immunoprecipitated by using the *Staphylococcus aureus* method (1, 13) with an excess of three different monoclonal antibodies specific for pAb412 and pAb416 (the SV40 large T antigen) and pAb421 (the cellular p53 antigen) (5). The immunoprecipitated proteins were fractionated on 10% sodium dodecyl sulfate-polyacrylamide gels (15). The gels were treated with En^{3}Hance (New England Nuclear Corp.), dried, and fluorographed. Lanes M correspond to ^{14}C markers of the indicated molecular sizes (in kilodaltons) obtained from Bethesda Research Laboratories, Inc. The positions of the SV40 large T and p53 antigens are indicated.

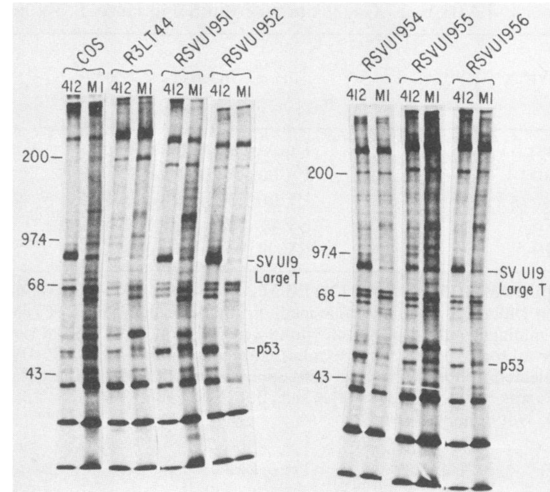

FIG. 2. Immunoprecipitation analysis of SV-U19 large-T-antigen-established cell lines. Cell lines established by either the mutant SVU19 large T antigen (RSVU1951, RSVU1952, RSVU1954, RSVU1955, and RSVU1956) or the Py large T antigen (R3LT44) were labeled with 150 μCi of [^{35}S]methionine for 17 h. Extracts were prepared with 100 mM Tris hydrochloride (pH 8.0)–100 mM NaCl–0.5% Nonidet P-40–1.6 TIU of aprotinin per ml, and equal amounts of acid-precipitable radioactivity (7.8 × 10^6 cpm) were immunoprecipitated by using a modified *S. aureus* method (1, 13) that used protein A-Sepharose (Pharmacia, Inc.) with an excess of monoclonal antibodies specific for pAb412 and pAbMI, (adenovirus 5 region EIa proteins) (6). The molecular sizes of ^{14}C markers (in kilodaltons) and the positions of the SV-U19 large T and p53 antigens are indicated.

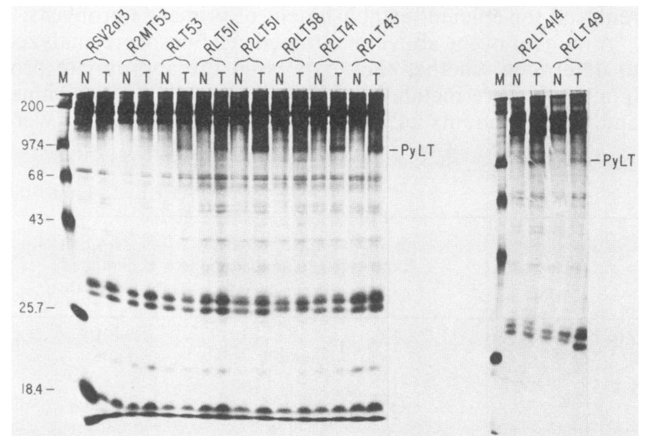

FIG. 3. Immunoprecipitation analysis of Py large-T-antigen- and middle-T-antigen-established cell lines. In this analysis, cell lines established by either the Py large T antigen (RLT53, RLT511, R2LT51, R2LT58, R2LT41, R2LT43, R2LT49, and R2LT414), the Py middle T antigen (R2MT53), or the control retrovirus (RSV2a13) were labeled with 200 μCi of [^{35}S]methionine for 18 h. Proteins were extracted, and equal amounts of acid-precipitable radioactivity (7.2 × 10^6 cpm) were immunoprecipitated with anti-Py T-antigen serum (lanes T) or nonimmune rat serum (lanes N) as described previously (9, 10). The immunoprecipitated proteins were fractionated on 10% sodium dodecyl sulfate-polyacrylamide gels. The molecular sizes of the ^{14}C markers (in kilodaltons) (lanes M) are indicated on the left, and the position of the Py large T antigen (PyLT) is indicated on the right.

TABLE 3. Tumorigenicity of immortalized REF cell lines[a]

Virus	Cell line	Tumorigenicity in nude mice (no. of tumors/no. of injections)
Zip Neo SV(X)I (Backbone)	RSV2a13	0/2
MT-5 (Py middle T antigen)	R2MT53	2/2[b]
LT-4 (Py large T antigen)	R2LT41	0/2
	R2LT43	1/2[c]
	R3LT44	0/2
	R2LT414	0/2
LT-5 (Py large T antigen)	RLT53	0/4
	RLT54	0/2
	RLT511	0/2
	R2LT51	0/2
	R2LT58	0/4
SV40-6 (SV40 large T antigen)	RSV4062	2/2[b]
	RSV4068	0/2
	RSV40611	0/2
	R2SV4061	0/2
	R2SV40613	0/2
SV-U19-5 (SV-U19 large T antigen)	RSVU1951	0/4
	RSVU1952	0/2
	RSVU1954	0/2
	RSVU1955	0/2
	RSVU1956	0/2
	RSVU1957	0/2

[a] The tumorigenicity of the cell lines was determined by subcutaneous injection of 10^6 cells in 0.1 ml of phosphate-buffered saline into 28- to 35-day-old nude mice (BALB/c/An Cr-Nu, obtained from the National Cancer Institute) irradiated with 500 rads 24 h previously to eliminate the natural killer cells. Cells were prepared for injection as described previously (16). Nude mice were observed for tumor formation every week for at least 12 weeks.
[b] Tumors were visible within 2 to 3 weeks.
[c] Tumor was visible after 10 weeks.

again, an extract prepared from the cell line RSV2a13 was included as a negative control. In addition, an extract prepared from R2MT53, the cell line isolated after infection with the Py middle T antigen recombinant retrovirus, was also analyzed. The results show that a protein which comigrates with the authentic Py large T antigen was readily detectable in the cell lines established after infection with the Py large T antigen recombinant retrovirus. In summary, these results show that all cell lines established by infection with recombinant retroviruses encoding the SV40 large T, SV-U19 large T, or Py large T antigen express the appropriate oncogene product.

We analyzed the tumorigenicity of many of these established cell lines by subcutaneous injection of 10^6 cells into irradiated nude mice. RSV2a13, the cell line isolated after infection with the retrovirus encoding only resistance to G418, was not tumorigenic, whereas R2MT53, the cell line isolated after infection with the retrovirus encoding the Py middle T antigen, was highly tumorigenic (Table 3). Both of these cell lines were established by passage through crisis. When cell lines derived from Py, wild-type SV40, or mutant SV-U19 large T antigen retroviruses were injected, only two cell lines, R2LT43 and RSV4062, were found to be tumorigenic. In the course of two challenges, R2LT43 yielded one tumor, which first became visible after a 10-week latency period, whereas RSV4062 yielded visible tumors within 2 to 3 weeks during both challenges. Nude mice injected with all the other large-T-antigen-established cell lines remained tumor free for 12 weeks. Neither of the two large-T-antigen-established cell lines that were tumorigenic could be distinguished from the others by their morphology, growth rate, level of T-antigen expression, or p53 expression.

The results presented above demonstrate that the large T antigens of both SV40 and Py are capable of efficiently establishing cell lines derived from REFs. In addition, we found that a fraction of the established cell lines, one of five and one of nine for SV40 large T and Py large T retroviruses, respectively, were tumorigenic upon injection into nude mice. Initially, this low incidence of oncogenic transformation with the SV40 large T antigen was very surprising, because it has previously been suggested that a high level of expression of the SV40 large T antigen in conjunction with an elevated level of p53 is sufficient for complete transformation (14; for a review, see reference 27). However, further experiments in which only SV40 large T antigen retroviruses were used to infect either mouse NIH 3T3 or rat F111 cells have shown that only a small fraction of the infected cells becomes fully transformed (11; unpublished data). Moreover, other experiments in which the provirus present in the complete transformants was rescued by superinfection with Moloney murine leukemia virus have shown that these low-frequency transformants are not due to mutant proviruses as a result of errors during reverse transcription. Therefore, further experiments are necessary to understand the mechanism by which the SV40 large T antigen gives rise to these oncogenic transformants.

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