The Resistance of SV40-Transformed 3T3 to Supertransformation (35355)

CHARLES D. SCHER, KENNETH K. TAKEMOTO, AND GEORGE J. TODARO

Viral Leukemia and Lymphoma Branch, National Cancer Institute, and Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014

The mouse cell line 3T3 has been transformed both singly and successively by polyoma virus and SV40 (1, 2). The doubly transformed cells, SVPY3T3, initially recognized by their ability to grow to a higher saturation density than polyoma-transformed lines, have been shown to have the T-antigens of both viruses (3). In addition, DNA–RNA hybridization studies have demonstrated the presence of both viral genomes (4, 5). Double transformation of SV40-transformed hamster cells by polyoma has also been described (6). The phenomenon of double transformation with two different oncogenic DNA viruses, polyoma and SV40, suggests the possibility of supertransformation of SV40-transformed cells with SV40. The reported presence of multiple copies of the SV40 genome in SV40-transformed cells (5) would be expected to provide regions of complementarity between the integrated and superinfecting virus; crossing over between the two could result in the integration of the superinfecting genome. Such events have been found to occur, at low frequency, with the temperature bacteriophage lambda (7, 8).

The present study was undertaken to learn if SV40-transformed 3T3 cells are resistant to supertransformation by this virus. Two approaches were made: (i) superinfection of lines of SV40-transformed 3T3 with low saturation density to determine whether cells with altered growth properties (further transformation) could be selected; and (ii) superinfection of SV40-transformed 3T3 with plaque mutants of SV40, followed by fusion with permissive cells to rescue a persistent superinfecting virus.

Methods. Virus strains. The small plaque (SV-S), minute plaque (SV-M), and temperature sensitive large plaque (SV-L) mutants of SV40 have been described (9). "Rescued" SV40 mutants were obtained by recovering the virus from transformed cell lines after fusion with African green monkey kidney cells (GMK). Twice "rescued" strains (SV-S-R^2, SV-M-R^2, and SV-L-R^2) retain the plaque morphology, host-restriction, and temperature sensitivity of the transforming mutant but have increased transformation efficiency (10). Wild type SV40, strain 776 (SV-776), containing both small and minute plaque viruses, was also used in certain experiments.

Cell lines. The origin and establishment of the mouse embryo line, 3T3, has been described (11). SV-71C and SV-74C are lines of 3T3 cloned after transformation by SV-L. UV-15-Cl-1 and UV-15-Cl-5 are lines of 3T3 cloned after transformation by UV-inactivated SV-S. Fl^{2}SV101 is a "flat variant" of the SV40-transformed line SV101 selected by Pollack *et al.* (12) for low saturation density by using FUdR to kill dividing cells. SV-3T3-52 and SV-3T3-262 are cloned SV-776 transformed lines of 3T3 with relatively low saturation densities. Primary GMK and the established simian cell line CV-1 were maintained and used for fusion and viral assay as previously described (13).

Cell culture. Dulbecco's modification of Eagle's medium supplemented with 10% calf serum (Colorado Serum Co.) was used in all experiments. 3T3 cultures were maintained in 50-mm plastic petri dishes and were subcultured using 0.1% trypsin in phosphate-buffered saline. Calf anti-SV40 antiserum was routinely added to the medium at all passages after infection.

Superinfection of SV-3T3. The transforma-

TABLE I. Superinfection with SV40 of Clonal Lines Derived from 3T3.[a]

Line	Transformed by	Saturation density (cells/plate × 10^6)	Detection of foci with altered saturation density[a]
SV-3T3-52	SV-776 (wild type)	6	—
SV-3T3-262	SV-776 (wild type)	4	—
UV-15-Cl-1	UV-inactivated SV-S	5	—
Fl²SV101	FUdR killing of SV-776-transformant	2	—
Py3T3-41	Polyoma virus	4	+
3T3 clone 42[b]	—	2	+

[a] Clonal lines were infected with either 4 × 10^9 PFU/ml of SV40 strain 776 or 1 × 10^9 PFU/ml of SV-S. Cell dilutions were made and the plates were stained with hematoxylin when confluence was reached.

[b] A subclone of 3T3 that was carried under culture conditions that favored the growth of variants with a higher saturation density. The standard culture of 3T3 clone 42 has a saturation density of 0.8–1.2 × 10^6 cells/plate.

tion of 3T3 with SV40 and the selection of transformed clones has been described (14). SV40-transformed lines, in the exponential phase of growth, were superinfected with SV40 in a smilar manner. The infected lines together with uninfected controls were trypsinized and cell dilutions were made. When confluence was attained, the plates were stained with hematoxylin to detect foci of higher saturation density than the parent line.

Recovery of the SV40 by cell fusion. After infection, lines were carried in mass culture for 1–2 months prior to fusion, being transferred when confluence was reached (generally at weekly intervals) at a 1:100 cell dilution. SV40 was recovered from transformed lines by fusion with permissive cells using UV-inactivated Sendai virus (13). Transformed (2–10 × 10^6 cells) and permissive (2–10 × 10^6 cells) cultures were fused at 37°. The cell layer and supernatant fluids were harvested 10–15 days later and stored at −70°.

Results. Attempt to demonstrate morphologically altered cells. The established cell line 3T3 has been useful in the study of viral transformation in part because of its low saturation density; SV40-transformed cells can be readily recognized by their ability to continue dividing under conditions where the control cells remain confined to the monolayer. Cloned lines of SV40-transformed 3T3 have different saturation densities ranging from 4 to 25 × 10^6 cells/plate. Supertransformation of SV40-transformed cloned lines which have relatively low saturation densities might result in cells which have acquired increased ability to grow under crowded culture conditions. Several SV40-transformed cloned lines with low saturation densities were infected with either 4 × 10^9 PFU/ml of SV-776 or 1 × 10^9 PFU/ml of SV-S. The infected cells were trypsinized and inoculated at concentrations of 1 × 10^4, 1 × 10^3, and 1 × 10^2 cells/plate. No foci with increased saturation density were detected (Table I). Clones of polyoma-transformed 3T3, with comparable saturation densities readily showed altered growth properties after infection by SV40 (2).

Superinfection of nonreleasing SV40-transformed lines. UV-15-Cl-1 and UV-15-Cl-5 are two cloned SV40-transformed lines obtained with UV-inactivated SV-S; neither clone yields infectious virus after fusion with permissive cells although both have SV40 T-antigen (Aaronson, S. A., unpublished data) and SV40 specific messenger RNA (15). The superinfection of these lines with a high input multiplicity of SV40 might yield SV40-supertransformed cells or persistently infected cells at a great enough frequency to be detected by fusion with permissive GMK. In a reconstruction experiment, UV-15-Cl-1 (2 × 10^6 cells) containing various numbers

TABLE II. Recovery of SV40 from Superinfected Mass Cultures of SV40-Transformed Nonreleasing Cloned Lines.

Superinfecting mutant	PFU/ml	Expected transformation frequency[a] (%)		PFU rescued[b]	
		UV-15-Cl-1	UV-15-Cl-5	UV-15-Cl-1	UV-15-Cl-5
SV-S-R²	7×10^8	≥25	≥25	0	0
SV-M-R²	3×10^7	7	2	0	0

[a] Assuming that the cloned lines are transformed as efficiently as 3T3.

[b] Titer of virus in 0.2 ml of fusion harvest assayed on GMK cells. If 0.1% releasing cells are added to a population of nonreleasers, SV40 can be recovered by fusion.

of SV-68C, a transformed clone of 3T3 which yields virus (SV-S) upon fusion with permissive cells, was fused with an equal amount of GMK. SV40 was detected when the population contained as little as 0.1% of the releasing line.

UV-15-Cl-1 (5×10^5 cells/plate) and UV-15-Cl-5 (1×10^6 cells/plate) in the exponential phase of growth were infected with plaque mutants of twice "rescued" SV40 (10). Still, no cells with altered growth properties were detected. After growth for 1 month, the lines were fused with permissive GMK. No infectious virus was recovered from either cell line (Table II). At the multiplicities of SV-S-R² used, the transformation frequency of nontransformed 3T3 would be as high as 25%.

Superinfection of cloned lines transformed by temperature sensitive SV40. Certain nonreleasing lines of mouse kidney cells transformed by UV-irradiated SV40 have been found by Dubbs and Kit to fuse poorly with permissive monkey cells (16). In order to rule out poor fusion as a cause for the inability to recover SV40, two known releasers were superinfected. SV-71C and SV-74C are SV40-transformed cloned lines obtained by the infection of 3T3 with SV-L; both lines yield high titers of SV-L upon fusion with GMK.

SV-71C and SV-74C (2×10^5 cells/plate) in the exponential phase of cell growth (saturation density 10–20 $\times 10^6$ cells/plate) were infected with either 5×10^8 PFU/ml of SV-S or 3×10^8 PFU/ml of SV-M. At this multiplicity, either mutant transforms at least 20% of 3T3 (10). After superinfection, no cells with altered growth properties could be observed. The superinfected cultures were maintained for 2 months. SV-71C cells, control and superinfected, were fused with GMK, harvested and assayed on GMK at 37 and 40° (Table III). All of the mass cultures released 10^4–10^5 PFU/ml of the large plaque type. Neither the superinfecting virus nor the original transforming temperature sensitive virus (SV-L) could be detected when the assay was carried out at 40°, the nonpermissive temperature for the growth of SV-L (9) except for one experiment where 11 PFU of SV-L were found. The temperature sensitivity of SV-L is known to be "leaky" (17).

The superinfected lines of SV-74C were fused with the established monkey cell line CV-1. Growth of SV-L, but not SV-S or SV-M, is restricted in CV-1 (17). Again, all of the mass cultures released 10^4–10^5 PFU/ml of the large plaque virus. When assayed at 40° on GMK cells, no virus could be detected in either of the superinfected lines (Table III). There was no more than a fourfold decrease in the titer of original input SV-S or SV-M when they were assayed at 40°. Thus under conditions where fusion and release of infectious virus are known to occur, the superinfecting virus was not recovered.

Discussion. The supertransformation of SV40-transformed 3T3 could not be demonstrated using as criteria: (i) altered cellular growth properties, and (ii) rescue of supertransforming virus. Several SV40-transformed cloned lines with low saturation densities were superinfected with SV40 in an attempt to demonstrate cells with altered growth properties. No cells with higher saturation densities than the parent lines were detected

TABLE III. Recovery of SV40 from Superinfected Cultures of SV40-Transformed Clones.

Clonal line	Superinfecting mutant	Permissive cells used for fusion	PFU and plaque type of rescued virus assayed at:[a]	
			37°	40°
SV-71C[b]	SV-S	GMK	3×10^3 SV-L	0
	SV-M	GMK	2×10^3 SV-L	11 SV-L
	None	GMK	3×10^4 SV-L	0
SV-74C[b]	SV-S	CV-1	5×10^3 SV-L	0
	SV-M	CV-1	2×10^3 SV-L	0
	None	CV-1	2×10^4 SV-L	0
Stock SV-S			2×10^7 SV-S	5×10^6 SV-S
SV-M			9×10^5 SV-M	6×10^6 SV-M

[a] Titer of virus in 0.2 ml of fusion harvest assayed on GMK.
[b] Transformed by SV-L.

even though the conditions used would have readily selected for such a cell if it were present.

Superinfecting virus could not be recovered from four cloned lines of SV40-transformed 3T3 exposed to high titers of SV40. Rescue of two plaque mutants of SV40 from a cloned transformed line of 3T3 simultaneously infected with both has been demonstrated by Dubbs and Kit (18). This is evidence that after fusion, the DNA of both can undergo replication with the formation of infectious virus. Since two viral genomes can be rescued from the same cell, the absence of recoverable superinfecting virus in the experiments described here suggests that the second virus is unable to become permanently associated with the host cell once transformation has become fixed. The permanent association of the superinfecting genome required the adsorption, penetration and uncoating of the superinfecting virus prior to fixation of the viral genome. These early functions may be altered in an SV40-transformed cell (19). On the other hand, another step may be involved; the presence of "repressor" substances (20) in an SV40-transformed cell might prevent the transcription of the superinfecting genome and perhaps its integration. Transformation by SV40 may depend upon site specific integration mechanisms as has been demonstrated for the temperate bacteriophages (21). If the specific site is occupied, the integration of another SV40 genome may be prohibited. Studies are in progress to determine which of these early functions are altered in SV40-transformed 3T3. It is, of course, possible that the superinfecting virus does integrate but that it neither affects cellular growth nor is it accessible to "rescue."

The methods used in the present study were capable of detecting the presence of small amounts of the rescued superinfecting mutants. The inability to detect the superinfecting virus after infection of SV40-transformed cells with high titers of SV40, as well as the absence of cells with altered growth properties, suggests that if supertransformation occurs it is an uncommon event.

Summary. Attempts were made to learn if SV40-transformed cells are resistant to supertransformation by infecting SV40-transformed clones of 3T3 with SV40. No alteration of growth properties was detected after SV40 infection of four cloned lines of SV40-transformed 3T3 that had stable low saturation densities. In contrast, SV40 infection of polyoma-transformed 3T3 having comparable saturation densities yielded easily detectable double transformants. Two lines of 3T3, which had been transformed by UV-inactivated SV40 and did not release virus upon fusion with permissive cells, were superinfected with SV40. After dilution of residual virus by cell growth, the superinfecting virus could not be recovered from either of the cell lines upon fusion with permissive (GMK) cells. Two lines transformed by a temperature sensitive large plaque mutant of SV40 were infected with temperature resis-

tant small and minute plaque mutants. Fusion of the cells with GMK at the permissive temperature was effective in releasing the initial transforming mutant from both clones, but no superinfecting (temperature resistant) virus could be detected at either the permissive or nonpermissive temperature. We conclude that if supertransformation occurs with SV40 it is an uncommon event, for superinfecting virus could not be recovered, nor could cells with altered growth properties be detected.

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Received Oct. 6, 1970. P.S.E.B.M., 1971, Vol. 136.