

"RESCUED" SV40: INCREASED TRANSFORMING EFFICIENCY IN MOUSE AND HUMAN CELLS

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Abstract.—Plaque-size mutants of simian virus 40 (SV40) have been characterized with respect to their ability to transform mouse and human cells *in vitro*. Large plaque mutants of SV40 were extremely inefficient at transforming mouse or human cells, requiring approximately 50 times more infectious particles per transformation event than did the small plaque mutants. The minute plaque SV40 was the most efficient, requiring less than 10^3 plaque-forming units per transformation in mouse Balb/3T3 cells and only 1.5×10^4 plaque-forming units per transformation unit in the "susceptible" human strains. Both small and minute plaque virus were unable to help the large plaque virus to transform. Virus "rescued" from transformed 3T3 cells by cocultivation with monkey kidney cells was more efficient at transformation than the parental type. The increased efficiency could be shown for each plaque type and in human as well as in mouse cells. The property of "high efficiency transformation" is stable through at least two passages in monkey kidney cells.

Three different plaque-size mutants of simian virus 40 have been described.¹ They are found in wild-type virus populations and have also been recovered from SV40-transformed cells and SV40-induced tumor cells by the technique of cell fusion with monkey kidney cells.² The present study is concerned with the transforming ability of virus that has been "rescued" from the transformed cells. The continuous mouse cell lines 3T3³ and Balb/3T3⁴ and certain "susceptible" human diploid cell strains⁵ were used to assay for transformation.

The results show that there are pronounced differences in the transforming ability of the SV40 mutants in both mouse cells and human cells. The minute plaque virus is the most efficient in transformation, and the large plaque virus is by far the least efficient. While many of the properties of the rescued virus of each plaque type are indistinguishable from those of the parental virus,^{2, 6} it is demonstrated that rescued virus is more efficient in transformation.

Materials and Methods.—*Mutants of simian virus 40.* The large plaque and small plaque mutants of SV40 have been described previously.¹ Wild type strain 776 was used in certain experiments. This virus consisted of a mixture of 70% small plaque and 30% minute plaque types. Minute plaque virus was isolated and plaque purified from strain 776. Primary cultures of African green monkey kidney cells were used to assay SV40 infectivity. Stock virus pools were grown in these cells in Blake bottles. The small and large plaque standard pools each had a titer of 5×10^8 plaque-forming units per milliliter (PFU/ml). The minute plaque stock had a titer of 8.5×10^7 PFU/ml. For certain experiments, the large plaque stock was concentrated by centrifugation in a Spinco model L centrifuge at 100,000 rpm for 2 hours. The pellet was resuspended in $1/10$ th the original volume. The concentrated virus titered 5×10^9 PFU/ml.

Recovery of simian virus 40 by cell fusion: Transformed colonies produced by each of the three plaque mutants were picked and then recloned twice in the presence of rabbit anti-

SV40 antiserum. Cell extracts of these clones had no detectable infectious virus. Simian virus 40 was recovered from transformed cells by the technique of cell fusion using irradiated Sendai virus.^{7, 9} The method employed was the same as that previously described.²

Recovered virus, harvested 10–14 days after fusion, was tested for infectivity and for transformation. Its genetic stability was tested by passing the recovered virus stocks twice in African green monkey kidney cells and then retesting for infectivity and transforming efficiency.

Transforming activity of simian virus 40: The transformation assay with SV40 and mouse 3T3 cells has been described.¹⁰ The same methods were used for transformation of Balb/3T3. Logarithmically growing cultures containing $2-5 \times 10^5$ cells per plate were infected for 3 hr with each of the virus preparations. After the adsorption period the cell layer was washed, and the cells were trypsinized and reinoculated onto 50-mm plastic Petri dishes at varying densities, from 10^2 to 10^4 cells per plate. The medium used in all experiments was Dulbecco and Vogt's modification of Eagle's medium supplemented with 10% calf serum. The medium was changed twice a week when the cultures were sparse and three times a week when the cultures had approached confluence. The cultures were fixed and stained 16–18 days after infection, and the denser, darker-staining transformed colonies were counted under low power with a dissecting microscope.

The transformation assay with human diploid cells was essentially the same as described above except that the virus-infected cells were inoculated at densities ranging from 1×10^4 to 1×10^5 cells per plate and, after the adsorption period, were continuously maintained in the presence of rabbit anti-SV40 antiserum. Both normal and "susceptible" cell strains were initiated from forearm skin biopsies and tested before the tenth tissue culture passage.⁵ Because human cells grew somewhat more slowly than the mouse cells, the former were not fixed and stained until 18–20 days after infection.

Induction of simian virus 40 T-antigen: Human diploid cells and mouse 3T3 cells infected with SV40 were planted onto cover slips after the virus adsorption period and stained for the virus-specific T-antigen 48–72 hours after infection. Previous studies have shown this to be the peak of T-antigen production, in both human and mouse cells.^{4, 11} The indirect method with hamster ascitic fluid containing SV40-T antibody¹² and fluorescein-conjugated rabbit antihuman gamma globulin was used.

Results.—Table 1 shows the plaque-forming and the transforming units with the three SV40 mutants. The transforming units (the number of transformed colonies produced per 10^6 cells per milliliter of virus inoculum) were determined both in mouse Balb/3T3 cells and in two strains of susceptible human diploid cells (L. S. and B. L.). The ratio of plaque-forming units to transforming units is a measure of the efficiency of the particular virus stock at producing transformation. In various experiments with large plaque virus the ratio of plaque-

TABLE 1. Transformation efficiency of large, small, and minute plaque simian virus 40 on human and mouse cells.

1. Human diploid cells (strain L.S.)			
Virus	PFU/ml*	TFU/ml†	PFU/TFU
SV-large	5.0×10^8	1.8×10^2	2.8×10^6
SV-small	5.0×10^8	6.2×10^3	8.0×10^4
SV-minute	8.5×10^7	5.9×10^3	1.5×10^4
2. Mouse Balb/3T3 cells			
SV-large	5.0×10^8	2.6×10^3	1.9×10^5
SV-small	5.0×10^8	1.1×10^5	4.6×10^3
SV-minute	8.5×10^7	9.5×10^4	8.9×10^2

* Plaque-forming units/ml.

† Transforming units/ml.

forming units (PFU) to transforming units (TFU) was found to be $2-5 \times 10^5:1$ with Balb/3T3 cells and about ten times higher with "susceptible" human cells. With small plaque virus the ratio ranged from 2 to $8 \times 10^3:1$ in the mouse cells and again about tenfold higher with human cells. The minute plaque virus was the most efficient; it needed for transformation only a fifth as many infectious particles as were needed by small plaque virus. This efficiency of transformation is greater than has previously been reported for any oncogenic DNA-containing virus.

Previous studies of the infection of human and mouse cells with small plaque simian virus 40 have shown that the frequency of transformation is directly related to the number of cells producing SV40 T-antigen 48-72 hours after infection. The ratio of T-antigen positive cells to transformed colonies is 10:1 with mouse cells⁴ and about 200:1 with human cells.¹¹ When the different plaque-size mutants were tested for their ability to induce T-antigen in susceptible human cell strains, a parallel difference was found among the three plaque types. Large plaque virus was extremely inefficient at inducing SV40 T-antigen. With 5×10^9 PFU of large plaque virus (a multiplicity or infection of 10^4 to 1), less than 0.5 per cent of the cells were positive for T-antigen (Table 2). Small plaque virus again was roughly 100 times more efficient per plaque-forming unit.

Transforming ability of "rescued" virus: Since infectious simian virus 40 can be obtained from SV40-transformed lines by fusion with African green monkey kidney cells, the properties of the recovered virus can be compared with those of the original infecting virus. With each plaque type, transformants of 3T3 were produced. Lines that were twice cloned in SV40 antiserum were used to obtain "rescued" virus by the cell fusion technique. In all cases the plaque type, host range, and temperature sensitivity were found to be that of the original infecting virus.² The recovered virus after the first cycle was used to transform new 3T3 cells, and the procedure (identifying the transformed clones, isolating and re-cloning them, and recovering the virus by fusion) was repeated. The progeny virus of the second as well as that of the first cycle and the original virus were all simultaneously titered in African green monkey kidney cells and assayed for transforming ability in 3T3, Balb/3T3, and human cells. The results, expressed in terms of the number of plaque-forming units required to induce one transformed colony, are shown in Table 3.

In general, the recovered virus was more efficient at transforming than was the original parental virus. This was most pronounced with the small plaque and

TABLE 2. SV40 T-antigen induction in susceptible human cells (strain B. L.).

Virus	Titer (PFU/ml)	T-antigen positive (%)*
SV-large	5.0×10^9	0.43
SV-large	5.0×10^8	0.09
SV-large	5.0×10^7	<0.01
SV-small	5.0×10^8	12.0
SV-small	5.0×10^7	1.3
SV-776	1.0×10^9	22.3

* 48 hr after infection.

TABLE 3. *Efficiency of transformation of "rescued" SV40 in mouse and human cells.*

Virus	PFU/transforming Unit			Enhancement factor*
	Original	1st rescued	2nd rescued	
Experiment 1. Mouse 3T3 cells (clone 42)				
SV-large	1.2×10^6	6.5×10^6	6.3×10^5	2
SV-small	2.3×10^4	1.2×10^3	5.2×10^2	44
SV-minute	5.1×10^3	1.0×10^3	5.0×10^2	10
Experiment 2. Mouse Balb/3T3 cells (clone 31)				
SV-large	1.9×10^6	9.3×10^4	5.8×10^4	3
SV-small	4.6×10^3	1.5×10^3	4.2×10^2	11
SV-minute	8.9×10^2	1.9×10^2	1.3×10^2	7
Experiment 3. Human diploid cells (strains L. S. and B. L.)				
SV-large	2.8×10^6	2.4×10^6	1.4×10^5	20
SV-small	8.0×10^4	4.1×10^3	6.0×10^2	130
SV-minute	1.5×10^4	1.2×10^3	1.2×10^3	12

* PFU/TFU of second rescued virus.

PFU/TFU of original stock

least evident with the large plaque type. The degree of enhancement of large plaque virus with mouse cells may well not be significant. The "rescued" small plaque and minute plaque viruses showed enhanced transforming ability, not only in 3T3 but also in Balb/3T3 and in human cells. In most cases the difference in efficiency between the first and second cycle viruses was slight compared to the difference between parental and first-cycle viruses.

The differences in the transforming efficiency of these viruses were again paralleled by differences in their ability to induce T-antigen, as shown in Table 4. The viruses recovered from two clones transformed by large plaque virus and two clones transformed by small plaque virus were compared with the parental viruses for their ability to induce SV40 T-antigen in human cells. One particular small plaque virus, SV-S-68, a "second cycle" recovered virus, was passaged twice in primary monkey kidney cells and was then retested for transforming efficiency in mouse and human cells. This virus was found to retain its ability to transform at high efficiency; indeed, it was still 10-50 times more efficient than the parental small plaque virus.

Attempts to "help" large plaque virus transformation with the other plaque types: 3T3 cultures containing 2×10^5 cells per plate were simultaneously infected with 5×10^9 PFU of large plaque, 5×10^8 PFU of small plaque, and 3×10^8 PFU of minute plaque virus. Large plaque virus at this multiplicity transforms 2-3 per cent of the cells, while small and minute plaque viruses transform 20-25 per cent. Twenty-eight independent transformed clones were picked and isolated in the presence of SV40 antiserum. All of them were replanted at varying densities, so that only one colony per plate was obtained. The colonies were then grown up to mass culture and recloned once more in the presence of antiserum.

All the clones released virus. Some of them released the small plaque and others the minute, but none were found to release the large plaque virus. As controls in these experiments, cells from two clones transformed by the large plaque virus and previously shown to release this type of virus were included in

TABLE 4. SV40 T-antigen-inducing ability of original and "rescued" SV40 in human cells (strain L. S.).

Virus	Titer (PFU/ml)	T-antigen positive cells (%)
Original		
SV-large	5.0×10^9	0.40
Rescued*		
SV-6 (large)	5.0×10^9	3.2
SV-7 (large)	3.0×10^9	3.5
Original		
SV-small	5.0×10^7	1.3
Rescued†		
SV-68 (small)	7.5×10^6	14.5
SV-69 (small)	8.0×10^6	7.8
SV-68‡ (small)	1.5×10^7	10.8

* "First-cycle" virus.

† "Second-cycle" virus.

‡ After two passages in primary green monkey kidney cells.

the tests. In both instances large plaque virus was readily recovered. The failure to recover any large plaque virus from the transformants is especially striking, since the cells were infected with a multiplicity of over 10,000:1 with large plaque virus. It appears then that the more efficient small plaque and minute plaque viruses are not able to facilitate transformation by large plaque virus.

Discussion.—Recent data from a number of laboratories have clearly established that the SV40 genome persists in transformed cells. Gerber's procedure of overlaying tumor cells on indicator cells¹² resulted in the release of simian virus 40. The release of small amounts of infectious virus from transformed hamster cells was also reported by other laboratories.^{13, 14} In a later study Gerber⁷ used UV-irradiated Sendai virus to facilitate cell fusion and the release of SV40. Facilitation of SV40 recovery by UV-irradiated Sendai virus has been confirmed and extended by others.^{2, 8, 9, 15, 16}

The present experiments were designed to determine some of the properties of the rescued virus. The lytic properties of the recovered virus have been described previously.^{2, 17} The rescued viruses are indistinguishable from the parental viruses with regard to plaque type, temperature sensitivity, host range, and ability to induce cellular DNA synthesis. However, when these viruses were tested for their transforming ability, it was found that the rescued virus was more efficient than the parental virus. The enhanced efficiency of transformation was most marked for small plaque virus but was also evident for the minute type. Increased efficiency of rescued large plaque virus could be demonstrated in human cells but not in 3T3 or Balb/3T3 cells. In general, the enhanced efficiency was at least as great in the human as in the mouse cells.

The enhanced efficiency of transformation of the simian virus 40 recovered from transformed cells could be due to either (1) selection of more efficient transforming viruses from the original stock, or to (2) host-induced modification during passage of the transformed cell or during the "rescue" process. Since plaque-purified virus was used, the postulated heterogeneity for transformation in (1) would have had to occur during the time that the virus stock was grown. The second possibility, the modification of virus, could arise during the rescue process

if, in certain cases, the rescued virus incorporated a portion of the host DNA in place of, or in addition to, the viral genome. We have primarily studied virus that was obtained directly from the fusion experiment and that had gone through relatively few lytic cycles following "rescue." However, the increased transforming efficiency appears to be a stable genetic property of the rescued virus through at least two subsequent passages in monkey kidney cells. The possibility that the rescued virus will revert to original parental-type virus with respect to transforming efficiency after long-term propagation in monkey cells is currently under investigation. The physical properties of the "rescued" virus are being examined. These viruses could conceivably have an increased fraction of their DNA homologous with the host cell genome as determined by DNA-DNA or DNA-RNA hybridization studies.

It might be argued that the apparent increased efficiency of the rescued virus is due for some reason to the production of a larger fraction of "defective" particles, i.e., those incapable of infectivity but capable of transformation.¹⁸ If this were the case, one would expect to find a proportion of transformed clones produced by infection with "rescued" virus from which no infectious virus could be recovered by cell fusion. However, we have examined more than 15 clones transformed with rescued virus and have found that all released infectious virus after cocultivation. Apparently, then, defective viruses if present to any appreciable extent are not responsible for a significant fraction of the transformation events in mouse 3T3 cells. In fact, we have recovered virus from all the mouse clones we have investigated so far, which indicates that in a nonpermissive host (3T3) the full complement of viral DNA is present. Dubbs and Kit⁶ also reported that virus can be rescued from all their SV40 transformed mouse cells. However, if UV-inactivated SV40 is used, one can obtain transformed lines that fail to yield infectious virus.^{16, 19}

The results of the present experiments show that the large plaque mutant, though highly lytic and most efficient at replicating in African green monkey kidney cells, is the least efficient in transforming both mouse and human cells. In this respect it bears a superficial resemblance to the virulent mutants of temperature phages that have lost their ability to lysogenize.^{20, 21} Takemori *et al.*²² have recently described the isolation of adenovirus type 12 mutants that are highly cytotoxic for human cells and whose ability for transformation in hamster cells is decreased. Like large plaque mutants of SV40, they form large plaques in their natural host but have lost transforming ability when tested in a system where vegetative growth is limited or absent.

The small plaque virus, which constitutes the majority of most wild-type stocks, is roughly 50 times more efficient in transformation per plaque-forming unit than the large plaque virus. A third plaque mutant of simian virus 40 which has received relatively little attention, the minute plaque virus, is the most efficient in transformation with a (PFU/TFU) ratio of less than 1,000:1 in mouse Balb/3T3 cells and just over 10⁴:1 in "susceptible" human cell strains. Minute plaque-type viruses occur in wild-type strains of SV40 other than SV-776, but they may easily be overlooked because of their pinpoint plaque size and delayed appearance (16-18 days). While some properties of the large-type virus are known (i.e., it is temperature-sensitive and grows poorly in continuous lines of

monkey kidney cells such as CV-1²), little is known as yet about the minute plaque virus. Unlike the large, the minute plaque virus can grow efficiently in continuous monkey kidney lines, and it replicates in African green monkey kidney cells at 40°C. Other large plaque viruses have been isolated from small and minute stocks;²³ like the prototype virus they are highly inefficient at transformation, suggesting that this is a general property of large plaque SV40. For studies involving transformation in either mouse or human cells, the large plaque mutants of SV40 would therefore appear to be the least useful, in spite of the fact that they can easily be grown to high titers in green monkey kidney cells.

One possible reason for the inefficiency of transformation by large plaque mutants of simian virus 40 could be a defect in integrating ability, analogous to the *int*⁻ mutants of λ^{24} and of P22.²⁵ Large plaque mutants of simian virus 40 transform inefficiently, but the transformants are stable once formed. The simultaneous infection with a great excess of large plaque virus argues against an integration defect as the basis of its poor transformability. Minute and small mutants are unable to facilitate integration of the large type in the manner that, for example, wild-type lambda phage is able to allow integration-deficient mutants of lambda to lysogenize.²⁴

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