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Isolation of Double Lysogens from 3T3 Cells Transformed by Plaque Morphology Mutants of SV40*

D. R. Dubbs and Saul Kit

DIVISION OF BIOCHEMICAL VIROLOGY, BAYLOR COLLEGE OF MEDICINE, HOUSTON, TEXAS

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Abstract. Simian virus 40 (SV40) rescued from 3T3 lines transformed independently by fuzzy plaque strain, SV40 (mKS-U4), and small-clear plaque strain, SV40 (mKS-U88), resembled the virus used to initiate the transformation. When a mixture of the two viruses was used for transformation, both plaque types could be rescued. Two clonal lines, after fusion with CV-1 cells, yielded both plaque types of infectious centers, and a third clonal line, 3T3(4-88)G-1, yielded fuzzy and small-clear plaque types of infectious centers plus large-clear infectious centers resembling "wild-type" SV40 clone 307L. Two secondary and 14 tertiary clones of 3T3(4-88)G-1 were isolated, all of which yielded fuzzy plaque type and clear infectious centers after fusion with CV-1 cells. When these infectious centers were picked and replated on CV-1 cells, the fuzzy and small-clear plaque types of SV40, as well as a large-clear plaque type resembling parental SV40, were isolated.

We would like to present evidence that at least two kinds of SV40 genomes can be integrated in a single cell. In order to show this, we have employed two plaque morphology mutants of SV40 rescued from mouse kidney cell lines transformed by ultraviolet (UV)-irradiated SV40.[1] 3T3 cells were transformed with a mixture of SV40 (mKS-U4), which produces large fuzzy (f) plaques, and SV40 (mKS-U88), which produces small-clear (sc) plaques. After cloning the transformed cells, virus rescue experiments were performed. Infectious centers produced by heterokaryons of the transformed and susceptible cells were scored morphologically and the rescued virus populations were analyzed as to plaque morphology.

Materials and Methods. Cell cultures: 3T3, an established line of mouse cells,[2] and CV-1 cells, an established line of green monkey kidney cells,[3] were propagated in R5a medium containing 0.5% lactalbumin hydrolysate and 10% calf serum.[4] SV40-transformed 3T3 cells were subcultured at 4 to 7 day intervals using the same medium. SV40-transformed 3T3 cells were cloned in liquid medium without a feeder layer.[1] However, for the third cloning of 3T3(4-88)G, the trypsinized cells were plated in liquid medium and incubated overnight at 37° to permit attachment to the surface. Then the liquid medium was removed and replaced by R5a medium containing a 0.6% agar, 10% calf serum, and 3.5% fetal calf serum.

Virus: The SV40 strains were propagated and assayed in monolayer cultures of CV-1 cells as previously described.[5] SV40 (307L), the parental strain, produces medium-clear (mc) to large-clear (lc) plaques, 3–5 mm in diameter on CV-1 monolayers in 14 days. Some late developing plaques appear between 12 and 14 days,

but by 18 to 21 days nearly all plaques are 5 mm in diameter. No fuzzy plaques have been encountered in any titrations of SV40 (307L) in 4 years. SV40 (mKS-U4) and SV40 (mKS-U88) were isolated from mKS-U4 and mKS-U88 mouse kidney lines transformed by UV-irradiated SV40.[1] SV40 (mKS-U4) produces large, turbid plaques with fuzzy edges. No clear infectious centers were encountered in more than ten fusion or cocultivation experiments with mKS-U4, the cell line from which SV40 (mKS-U4) was rescued, nor have any clear plaques been observed in more than 65 assays of this virus (at least 3000 plaques counted). SV40 (mKS-U88) produces small-clear plaques (sc), 1 mm or less in diameter at 14 days which enlarge to about 3 mm in 21 days. The mKS-U88 cell line from which SV40 (mKS-U88) was rescued yielded only sc infectious centers in fusion experiments and the rescued virus produced only sc plaques during the first three passages after rescue. SV40 (mKS-U4) was used after three passages in CV-1, and SV40 (mKS-U88) after two passages in CV-1 cells following rescue.

Transformation of 3T3 cells: Monolayer cultures of 3T3 cells were inoculated with parental SV40 (675 PFU/cell), SV40 (mKS-U4) (500 PFU/cell), or SV40 (mKS-U88) (167 PFU/cell), or with a mixture of two of the viruses. The cells were subcultured 24–48 hr after infection and the medium was changed at 4 to 7 day intervals until monolayers were complete (10–14 days). Thereafter, they were subcultured at 4 to 7 day intervals. 3T3 lines transformed by SV40 (mKS-U4) and SV40 (mKS-U88) are designated 3T3(U4) and 3T3(U88), respectively. Those lines transformed by a mixture of SV40 (mKS-U4) and SV40 (mKS-U88) are designated 3T3 (4-88). All of the transformed lines contained SV40 T-antigen. Infectious SV40 could not be recovered from cell-free extracts of any of the transformed lines.[6]

T-antigen: SV40 T-antigen was demonstrated either by complement fixation as previously described[7] or by immunofluorescence.[8] Acetone-fixed cover slips of the transformed cells were stained by the indirect method using antisera from SV40 tumor-bearing hamsters and anti-hamster gamma globulin prepared in goats and conjugated with fluorescein isothiocyanate.[8]

Rescue of SV40: SV40-transformed cells (5×10^6) were mixed with CV-1 cells (10×10^6) and treated with UV-irradiated Sendai virus (8000 hemagglutinating units) as previously described.[1] Frequencies of SV40-induction were determined by plating UV-Sendai treated mixtures of transformed and CV-1 cells in 60-mm plastic dishes containing 1×10^6 freshly trypsinized CV-1 cells. The cultures were incubated overnight to permit attachment, the liquid was removed, and an agar overlay was applied. Infectious centers were scored as to morphology and then were picked, assayed on CV-1 monolayers, and the plaque morphology of the progeny virus was scored. For further purification, plaques picked from secondary and tertiary plates were replated on CV-1 monolayers.

Results. Transformation of 3T3 with plaque morphology mutants of SV40: Five lines of 3T3 transformed by SV40 (mKS-U4) and four lines transformed by SV40 (mKS-U88) were isolated. As can be seen from Table 1, SV40 could be rescued very easily from all nine lines after fusion with CV-1 cells in the presence of UV-irradiated Sendai virus. The infectious centers produced by heterokaryons of transformed cell line 3T3(U4) and CV-1 cells were large and turbid with fuzzy edges (f) similar to those produced by infecting CV-1 cells with virus strain SV40 (mKS-U4), or by heterokaryons of the transformed line mKS-U4 from which the SV40 plaque mutant was derived.[1] On the other hand, infectious centers produced in fusion experiments by 3T3(U88) lines were small and clear (sc) and uniform in size, resembling the plaque morphology of SV40 (mKS-U88) in CV-1 or the infectious centers produced by heterokaryons of mKS-U88 cells. These results confirm previous reports that SV40 rescued from transformed cells has properties characteristic of the virus used to transform the cells.[6, 9]

TABLE 1. *Rescue of plaque morphology strains of SV40 from 3T3 cells transformed by infection with one or more plaque types.*

Virus used for transformation	Total lines studied	Average number infectious centers formed after plating 10^5 transformed cells	Morphology of Infectious Centers (%) f[a]	sc	lc
SV40 (mKS-U4)	5	18	100	0[b]	0
SV40 (mKS-U88)	4	16	0	100	0
Parental SV40 plus SV40 (mKS-U4)	4	22	0	0	+
	1	9	+	0	+
Parental SV40 plus SV40 (mKS-U88)	4	32	0	~25	~75
SV40 (mKS-U4) plus SV40 (mKS-U88)	15	8	2	98	0
		15	2	83	15
		28	1	49	50
		19	10	45	45
		3	41	18	41
		37	83	17	0

[a] f, sc, and lc signify fuzzy, small-clear, and large-clear type infectious centers, respectively.
[b] "+" indicates infectious centers of indicated morphology were present. "0" indicates infectious centers of indicated morphology were absent.

Transformation of 3T3 cells with mixtures of SV40 viruses: Of five lines of 3T3 cells transformed by a mixture of parental SV40 and SV40 (mKS-U4), only one line yielded virus of an f plaque morphology characteristic of SV40 (mKS-U4). When parental SV40 and SV40 (mKS-U88) were used to transform 3T3 cells, about 25 per cent of the heterokaryons produced sc infectious centers, the majority being lc.

A total of 15 lines of transformed 3T3 cells were isolated from cultures transformed with a mixture of SV40 (mKS-U4) and SV40 (mKS-U88). After heterokaryon formation with CV-1 cells, all 15 transformed cell lines yielded infectious centers of both the f and the sc type. In addition, eight of the uncloned lines yielded infectious centers of the lc type, which is typical of parental virus. The results with six selected 3T3(4-88) lines are shown in Table 1. Infectious centers produced by heterokaryons of three uncloned 3T3(4-88) lines with CV-1 cells were picked and the progeny virus scored for plaque morphology on CV-1 cells. In many instances, the lc infectious centers contained progeny virus of both f and clear plaque types. This is to be expected since the heterokaryons may contain up to 20 transformed nuclei. Some of these nuclei could be from cells transformed independently by SV40 (mKS-U4) or SV40 (mKS-U88). If both types of cells are activated in the same heterokaryon, the infectious center would be expected to contain both kinds of virus.

For further purification, plaques were picked from secondary plates and replated on CV-1 monolayers. Using this procedure f and clear plaque types of virus were isolated from heterokaryons of uncloned 3T3(4-88) cell lines. The clear plaques varied in size and many were significantly larger than those obtained with SV40 (mKS-U88).

Rescue of SV40 from clonal lines of 3T3(4-88): The previous experiment demonstrated that both f and clear plaque morphology mutants could be rescued from 3T3 populations transformed by a mixture of the two viruses. To learn whether individual cells in the doubly transformed population contained both

viral genomes, clones of 3T3(4-88) cells were isolated and the plaque morphologies of rescued virus were studied.

3T3(4-88) cells had a very low plating efficiency in liquid medium (0.2–1.0%). However, clones were isolated from plates where only one or two colonies appeared. The frequency of induction and the morphology of infectious centers produced by heterokaryons of selected clonal 3T3(4-88) lines and CV-1 cells are shown in Table 2. Heterokaryons of clonal lines of 3T3(4-88)B, 3T3(4-88)E, and 3T3(4-88)J produced infectious centers which were sc and of uniform size, and virus rescued from these clonal lines was of the sc morphology.

TABLE 2. *Rescue of plaque morphology mutants of SV40 from clonal lines of 3T3 cells transformed by infection with two plaque types.*

Parental Lines				Clonal Lines				
	Morphology of Infectious Centers (%)				Average number infectious centers formed after plating 10^5 transformed cells	Morphology of Infectious Centers (%)		
Line 3T3(4-88)	f[a]	sc	lc	Line 3T3(4-88)		f[a]	sc	lc
A	75	25	0	A-1	10	100	0	0
B	2	98	0	B-8	24	0	100	0
				B-9	0.2	0	100	0
				B-10	33	0	100	0
C	83	17	0	C-2	19	86	14	0
				C-2a[b]	53	70	30	0
				C-2b	31	60	40	0
				C-2c	61	47	53	0
				C-2d	46	+[c]	+	0
				C-2e	66	65	35	0
				C-4	7	100	0	0
				C-5	64	100	0	0
E	2	82	16	E-1	40	0	100	0
G	41	18	41	G-1	31	37	36	27
H(2)	10	45	45	H(2)-2	72	1	99	0
				H(2)-4	14	86	14	0
J	<1	50	50	J-1	12	0	100	0
				J-2	74	0	100	0
				J-3	TMC[d]	0	100	0

[a] f, sc, and lc signify fuzzy, small-clear, and large-clear type infectious centers, respectively.
[b] Secondary clones isolated from clonal line C-2.
[c] "+" indicates infectious centers of indicated morphology were present.
[d] TMC: Too many to count.

In contrast, heterokaryons of clones isolated from 3T3(4-88)A and from two clonal lines isolated from 3T3(4-88)C produced only f infectious centers. Of particular interest were clonal lines 3T3(4-88)C-2, 3T3(4-88)H(2)-4, and 3T3 (4-88)G-1 which yielded both f and clear infectious centers after fusion with CV-1 cells. In the case of 3T3(4-88)G-1, the clear infectious centers varied from sc, typical of SV40 (mKS-U88), to lc, typical of parental SV40. Also, many atypical infectious centers were encountered with clear centers and ragged edges. The clear and f infectious centers from 3T3(4-88)G-1 were picked and replated on CV-1 monolayers. With very few exceptions, typical f infectious centers yielded only f type SV40. The sc infectious centers occasionally yielded both f and sc virus populations. However, the large atypical infectious centers with ragged edges usually contained a mixture of f and clear plaque types. When

plaques were picked from the secondary plates, f plaques produced only f plaque type virus, and clear plaques yielded only clear plaque virus. In contrast to 3T3(4-88)B, 3T3(4-88)E, and 3T3(4-88)J from which only sc virus could be rescued, the clear plaque virus rescued from 3T3(4-88)G-1 produced clear plaques of varying size.

Eleven secondary clones of 3T3(4-88)G-1 were isolated. Of these, nine failed to yield any virus in two to five fusion trials. However, two of the secondary clones, 3T3(4-88)G-1h and 3T3(4-88)G-1t, yielded both f and clear infectious centers after fusion with CV-1 cells (Table 3). When these infectious centers were picked and replated on CV-1 monolayers, virus populations were obtained of the f and clear plaque morphology.

TABLE 3. *Rescue of plaque morphology strains of SV40 from the parental and clonal lines of 3T3(4-88)G.*

Cell line designation	Number of times cloned[a]	Average number infectious centers formed after plating 10^5 transformed cells	Morphology of Infectious Centers[f] (%)	
			f[b]	Clear
3T3(4-88)G	0	3	41	59
3T3(4-88)G-1	1	31	37	63
3T3(4-88)G-1h	2[c]	68	33	67
G-1t	2	43	20	80
3T3(4-88)G-1h-1	3	39	20	80
h-3	3	8	+[d]	+
h-4	3	21	31	69
h-6	3	15	36	64
h-8	3	17	32	68
h-9	3	22	+	+
h-10	3	33	27	73
3T3(4-88)G-1t-1	3	41	28	72
t-3	3	57	+	+
t-4	3	41	+	+
t-5	3	9	+	+
t-7	3	16	+	+
t-8	3	34	26	74
t-10	3	TMC[e]	+	+

[a] The first and second cloning was performed in liquid medium; the third cloning was performed under agar.

[b] f signifies fuzzy type infectious centers.

[c] Nine other secondary clones of 3T3(4-88)G-1 failed to yield virus in two to five fusion trials.

[d] "+" indicates infectious centers of indicated morphology were present.

[e] TMC: Too many to count.

[f] At least 75 infectious centers were scored for each cell line, and 9 to 42 infectious centers were picked from each clonal line and plated on CV-1 monolayers. Both *f* and clear plaque-type virus were isolated from all lines except 3T3(4-88)G-1h-3, 3T3(4-88)G-1h-9, and 3T3(4-88)G-1h-10.

To insure that the clonal populations were derived from single cells, 3T3(4-88)-G-1h and 3T3(4-88)G-1t lines were cloned a third time under an agar overlay to prevent migration of cells. Seven tertiary clones of each were isolated, and all yielded both f and clear infectious centers after fusion with CV-1 cells. Selected f and clear infectious centers (an average of 11 infectious centers per clonal line) were picked and replated on CV-1 monolayers. Progeny virus of both f and clear plaque types were obtained. The clear plaque virus rescued

from the secondary and tertiary clones produced clear plaques of variable size similar to those produced by clear plaque virus rescued from 3T3(4-88)G-1.

The situation was quite different, however, with the five secondary clones of 3T3(4-88)C-2. All five secondary clones yielded SV40 in rescue trials. Heterokaryons of the 3T3(4-88)C-2 secondary clones yielded two types of infectious centers—f and sc. In addition, many infectious centers encountered were atypical with clear centers and ragged edges. Approximately 100 sc and atypical infectious centers were picked from fusion experiments. However, all of these infectious centers yielded only progeny virus of the f morphology. It is possible that the f type virus outgrows the sc virus picked from the infectious centers, and therefore, the sc virus was not detected by plaque assay at terminal dilutions. The secondary clones of 3T3(4-88)C-2 might contain a defective or partially defective variant of SV40 (mKS-U88) capable of complementing SV40 (mKS-U4) to yield sc or ragged infectious centers instead of the f type. This variant may replicate poorly in CV-1 cells or be unable to replicate unless the cell is infected simultaneously by SV40 (mKS-U4).

Discussion. Double lysogens have been obtained by transforming 3T3 cells with two distinct plaque morphology mutants of SV40. Of prime importance in the successful isolation was the frequency of induction test employed for the rescue of SV40 from heterokaryons of the double lysogens and CV-1 cells. This test permitted the direct observation of infectious centers produced by heterokaryons, making it possible to select infectious centers with the desired morphology for further plating and characterization of progeny virus. Hence, it was possible to recover the sc virus by picking small infectious centers and to rescue f virus by picking fuzzy infectious centers. It was much more difficult to isolate the sc virus from infectious centers containing f, lc, and sc plaque types. Moreover, since the titers obtained with the f and lc virus are usually 10–100 times higher than those obtained with the sc virus, it would be difficult to isolate the sc virus from mixtures in *bottle cultures* since the f and lc types outgrow the sc, and the sc would not be detectable at terminal dilutions (plates with 1 to 100 plaques).

Although the present experiments provide evidence for the integration of two distinct SV40 plaque types into a single cell, they do not clarify the mechanisms of integration, the number of integration sites, or the average number of SV40 DNA equivalents integrated per cell.[10] In a diploid cell, it is reasonable to assume that there are at least two integration sites. Either integration of f and sc genomes independently at each of the sites or sequential integration at one of the sites might occur. Another possibility is that double length circular forms generated from SV40 (mKS-U4) and SV40 (mKS-U88) DNA's are integrated in tandem with or without secondary integration of SV40 (mKS-U4) and SV40 (mKS-U88) DNA's. Multiple length rings of phage S13 and ϕX174 replicative forms have been demonstrated and suggested as possible intermediates in recombination.[11] Multiple length rings of polyoma DNA have also been found after induction of ts-a mutants by temperature shift.[12]

A less attractive possibility is that the double lysogen originated during growth by spontaneous fusion of two single lysogens carrying the SV40 (mKS-U4)

and SV40 (mKS-U88) genomes, respectively, followed by cell hybridization. Usually, rather stringent selective methods must be used to isolate hybrid cells from the parental populations. The double lysogen 3T3(4-88)G-1 apparently grows as well as but no better than the single lysogens 3T3(U4) and 3T3(U88). It therefore seems improbable that one clone isolated from 3T3(4-88)G line happened to originate from a hybrid cell. However, this technique may be useful in obtaining a double lysogen by hybridization of two single lysogens which carry metabolic deficiencies which would permit selection of the hybrid double lysogen.

After cloning the double lysogen 3T3(4-88)G three times, both f and clear-type infectious centers were produced in fusion experiments with susceptible CV-1 cells. Many of the infectious centers yielded only one plaque type of virus when replated on CV-1 cells. This suggests that in some of the double lysogens, only one type of integrated genome was activated after heterokaryon formation with CV-1 cells. Had both f and sc type genomes been activated in every instance, probably only lc infectious centers would have been produced.

Some of the clear infectious centers produced by heterokaryons derived from clonal lines of 3T3(4-88)G were of the lc type. These often yielded both f and sc type plaques. In addition, virus picked from the lc infectious centers produced intermediate size clear (mc) and lc plaques. The origin of the mc and lc plaque types is not immediately clear. The sc plaque type of SV40 (mKS-U88) appears to be relatively unstable, and the plaque size increases when the virus is repeatedly passed in CV-1 cells at terminal dilutions. Hence, the mc and lc plaque types may be variants of the sc plaque type. It should be emphasized, however, that no mc or lc infectious centers have been observed in rescue experiments with clonal lines of 3T3(4-88)B, 3T3(4-88)E, and 3T3(4-88)J. Only the sc type virus has been rescued from these cell lines.

Alternatively, the mc and lc plaque types may have originated by recombination between the sc and f plaque types. If double-length circular forms generated from SV40 (mKS-U4) and SV40 (mKS-U88) DNA's were integrated, SV40 (mKS-U4), SV40 (mKS-U88), and/or recombinant forms might be activated during rescue. It is clear that in heterokaryons, the sc and f plaque types can complement each other giving rise to lc infections centers. Triple heterokaryons of CV-1 cells and of mKS-U88 and mKS-U4 (the transformed cell lines from which the f and sc plaque mutants were rescued) also produce lc infectious centers from which only f and sc virus types can be isolated (unpublished experiments). No other markers (except plaque morphology) distinguish the f and sc viruses, so that the interpretation that the mc and lc plaque types originated through recombinational events must be viewed with great caution.

If the mc and lc plaque types are indeed variants of the sc plaque type, integration of f, sc, and mc or lc genomes at three independent sites is consistent with the rescue data. Alternatively, if the mc and lc plaque types represent recombinants, then a tandem integration of f and sc genomes is equally consistent.[13]

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