Rescue of Simian Virus 40 from Cell Lines Transformed at High and at Low Input Multiplicities by Unirradiated or Ultraviolet-irradiated Virus

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The relation between simian virus 40 (SV40) input multiplicity during transformation of primary mouse kidney cultures and the subsequent rescue of SV40 from clonal lines of transformed cells has been studied. Primary mouse kidney cultures were transformed with unirradiated SV40 at input multiplicities varying from 0.06 to 200 plaque-forming units (PFU)/cell or with SV40 irradiated with ultraviolet (UV) light to a survival of 0.04 to 0.01. All of the transformed lines contained the intranuclear SV40 T antigen, but cell-free extracts prepared from the transformed cell lines failed to yield infectious virus when assayed on monkey kidney cell (CV-1) monolayers. After fusion with susceptible CV-1 cells induced by UV-inactivated Sendai, all of the lines transformed by unirradiated virus yielded infectious SV40. The frequency of induction and the incidence of successful trials did not depend on the multiplicity of infection. "Good" yielders were obtained from mouse kidney cells transformed at the low input multiplicity of 0.06 PFU/cell. In contrast, only 4 of 12 clonal lines transformed at moderately low input multiplicity, and none of the lines transformed at very low input multiplicity with UV-irradiated virus yielded infectious SV40. The four positive lines have been classified as "poor" or "rare" yielders.

Mouse kidney cell lines (mKS-U) transformed at high input multiplicities by ultraviolet (UV)-irradiated simian virus 40 (SV40) have been classified as nonyielders and as rare, poor, average, and good yielders on the basis of the incidence of successful rescue trials, the size of the SV40 yields, and the frequency of induction [the number of infectious centers formed when mixtures of transformed and susceptible CV-1 cells treated with UV-inactivated Sendai (UV-Sendai) were plated with freshly trypsinized CV-1 cells (7)]. Virus was rescued from rare yielders only once or twice in many trials and from poor yielders in less than half of the trials and, in each case, in very low yields. In contrast, virus was recovered in relatively good yields in all trials from average and good yielder lines. Once rescued, the SV40 strains recovered from good, average, poor, or rare yielders expressed all known SV40 functions during productive infection of CV-1 cells at 37 C (S. Kit et al., *Bacteriol. Proc.*, p. 157, 1969).

We considered the possibility that rare yielders differ from good or average yielder lines in the number of SV40 genomes integrated per cell. Rare yielders may contain only one type of SV40 genome integrated at a single site, whereas good and

average yielders may have one or more types of SV40 genomes integrated at several sites or at one site in tandem (4; D. R. Dubbs and S. Kit, *Bacteriol. Proc.*, p. 155, 1969). The probability of rescue might be greater in multiply transformed cells than in cells transformed by a single virus particle.

As a partial test of this hypothesis, four groups of primary mouse kidney cultures were transformed by unirradiated SV40 at both high and low input multiplicities. In addition, five groups of cell cultures were transformed at various input multiplicities by SV40 irradiated with UV light to a survival of 0.04 to 0.01. It was postulated that integration of more than one SV40 genome would be unlikely if the input multiplicity were very low. Rescue experiments were then performed on the transformed cell lines and on clonal lines selected from each group.

MATERIALS AND METHODS

Cell lines. Primary mouse kidney cell cultures were prepared from 10- to 14-day-old Swiss mice (Texas Inbred Mice Co., Houston, Tex.). Six-day-old cultures containing 12×10^6 cells were transformed by infection with unirradiated SV40 or with SV40 irradiated

with UV light to a survival of 0.04 to 0.01. The cells (mKS) transformed by unirradiated virus and the cells (mKS-U) transformed by UV-irradiated virus were grown in monolayer culture and were subcultured twice weekly (11). Transformed cell lines were cloned at passages 6 to 8 in medium containing 1% SV40 antiserum as previously described (8). All transformed cell lines contained SV40 T antigen as determined by complement fixation (CF) and immunofluorescence (IF). Cell-free extracts prepared from each transformed cell line failed to yield infectious SV40 when assayed on CV-1 monolayers.

CV-1 cells, an established line of green monkey kidney cells (9), were used as indicator cells. All cell lines were grown in R5a medium containing 0.5% lactalbumin hydrolysate and 10% calf serum (6).

Virus. SV40 clone 307L was grown and assayed in monolayer cultures of CV-1 cells (11). Sendai virus was grown in the allantoic cavity of chick embryos and concentrated 10-fold as previously described (7).

CF tests. Preparation of cell extracts and demonstration of SV40 T antigen by the CF test have been described previously (9, 10).

IF. The indirect immunofluorescent technique was used to demonstrate the presence of SV40 T antigen. Cover slip cultures were washed free from growth medium with phosphate-buffered saline (PBS) and fixed in cold acetone for 10 min. They were stored at -20°C until use. The fixed monolayers were rehydrated in PBS and stained for 1 hr at 37°C with anti-serum obtained from hamsters bearing SV40 tumors. Excess serum was washed away, and the cells were stained with fluorescein-labeled goat anti-hamster serum. The cover slips were mounted in buffered glycerol and examined with a fluorescence microscope. The SV40 anti-tumor serum and the goat anti-hamster serum were kindly supplied by John Riggs, California State Department of Public Health, Berkeley, Calif.

Rescue of SV40 from mKS cells. The procedure used for rescue of SV40 from mKS cells has been described (7). Mixtures of transformed cells and CV-1 cells in a ratio of 1:2 were suspended in PBS containing UV-Sendai. A total of 8,000 hemagglutinating units of UV-Sendai was used for each 5×10^6 transformed cells. The mixtures were chilled at 4°C for 10 min and transferred to a reciprocal shaker-incubator for 20 min at 38°C . The cells were washed and resuspended in growth medium. To determine the frequency of SV40 induction, the cells were diluted serially and plated in 60-mm petri dishes along with 10^6 freshly trypsinized CV-1 cells. The cells were incubated overnight, and then an overlay consisting of R5a medium with 7.5% fetal calf serum and 1% agar was added. Ten days later, we added a second overlay consisting of R5a medium supplemented with 0.5% lactalbumin hydrolysate, 0.1% Yeastolate (Difco), 1% fetal calf serum, and 1% agar containing neutral red. Plaques were counted 15 to 25 days after plating.

RESULTS

Rescue of SV40 from mKS lines. Primary mouse kidney cell cultures were inoculated with SV40 at input multiplicities of 200, 50, 0.6, 0.06, or 0.006 plaque-forming units (PFU)/cell (Table 1). Pre-

vious studies have shown that the SV40 stocks contain about 20 particles per PFU (4) and that about half of the PFU are adsorbed to mouse kidney cells in 2 hr at 37°C (12). Thus, the majority of the cells that were infected at the two lowest input multiplicities probably received only one virus particle, whereas those infected at high multiplicities received many. The following day the cultures were trypsinized and each was passaged to two bottles. The cells were fed twice weekly. Many colonies were observed after 18 to 24 days in cultures from groups I to III. Group IV cultures exhibited one to four transformed colonies after 37 to 53 days. Group V did not form colonies for as long as 62 days. Each transformed culture was used to establish a cell line. All lines contained the SV40 T antigen, but cell-free extracts did not yield SV40. The recovery of SV40 after fusion of these mKS cells with CV-1 cells is shown in Table 1. The results are presented as the average number of plaques formed after plating the equivalent of 10^6 transformed cells with freshly trypsinized CV-1 cells.

Rescue of SV40 from clonal mKS lines. The transformed mKS cells were cloned and shown to

TABLE 1. *Rescue of SV40 from mouse kidney lines (mKS) transformed at various input multiplicities of unirradiated SV40**

Group	Cell line	Input multiplicity		T antigen		Avg no. of plaques formed after plating 10^6 trans-formed cells
		PFU/cell	PFU/culture	CFU per mg of protein	IF	
I	1	200	2.4×10^6	105	+	43
	2			59	+	41
	3			228	+	12
	4			94		6
	5			143		18
	6			87		91
II	1	50	6.9×10^6	144	+	57
	2			139	+	59
	3			93	+	41
	5			108		70
	6			158		13
III	1	6×10^{-1}	7.0×10^6	72	+	50
	2			90	+	80
	3			38	+	8
	4			42		19
	5			113		32
	6			63		0.2
IV	4	6×10^{-2}	7.2×10^6	168	+	19
	5			133	+	39

* Rescue trials were performed on mKS lines at passages 6 to 12.

be positive for the SV40 T antigen by CF and IF. Table 2 shows the recovery of SV40 after fusion of these clonal mKS lines with CV-1 cells. Virus was rescued from all of the transformed cell lines, and all but one were classified as either "good" or "average" yielders. Transformed line clone 6 of group III was classified as a "poor" yielder. Thus, the incidence of successful rescue trials and the frequency of induction did not depend on the multiplicity of infection. "Good" yielders were obtained from mKS cells transformed at the low input multiplicity of 0.06 PFU/cell.

Rescue of SV40 from mKS-U lines. The same dilutions of SV40 used above were irradiated with UV light for 5 min to a survival of 0.04 to 0.01 and inoculated onto mouse kidney monolayers. The input multiplicities of these groups (I-UV through V-UV), shown in Table 3, were 8, 2, 6×10^{-3} , 6×10^{-4} , and 4×10^{-5} PFU/cell, calculated as residual live viruses. Many colonies

were observed after 18 to 24 days in groups I- to III-UV. Cultures from group IV-UV and group V-UV exhibited two to six and one colony per culture, respectively, after 37 to 53 days. Cell lines were established from each culture and shown to contain SV40 T antigen. The rescue of SV40 after fusion of the parental mKS-U lines with CV-1 cells is also shown in Table 3.

Rescue of SV40 from clonal mKS-U lines. The transformed mKS-U lines were cloned and shown to contain SV40 T antigen. Table 4 shows the rescue of SV40 after fusion of the clonal mKS-U cells with CV-1 cells. Virus was rescued from four of six clonal lines transformed at the input multiplicity of 8 PFU/cell (residual live virus). However, only 4 of 24 clonal lines transformed at the lower input multiplicities with UV-irradiated virus yielded infectious SV40. The virus rescued from

TABLE 2. Rescue of SV40 from clonal lines of mouse kidney cells (mKS) transformed at various input multiplicities of unirradiated SV40^a

Group	Cell line	Input multiplicity (PFU/cell)	T antigen		Avg no. of plaques formed after plating, 10 ⁵ transformed cells ^b
			CFU/mg of protein	IF	
I	1	200	125	+	3(3)
	2		155	+	3(4)
	3		270	+	3(4)
	4		258	+	4(3)
	5		273	+	4(3)
	6		269	+	3(3)
II	1	50	220	+	33(5)
	2		238	+	46(2)
	3		122	+	22(2)
	5		108	+	55(2)
	6		120	+	27(4)
III	1	6×10^{-1}	255	+	3(2)
	2		253	+	24(2)
	3		257	+	39(2)
	4		277	+	24(2)
	5		309	+	21(2)
	6		188	+	0.4; 0.06; 0; 0(4)
IV	4a	6×10^{-2}	222	+	5(3)
	4b		59	+	1(2)
	4c		311	+	51(2)
	5a		228	+	18(2)
	5b		106	+	24(2)
	5c		117	+	46(2)

^a Rescue trials were performed on clonal lines of mKS cells at passages 8 to 35.

^b Numbers in parentheses signify number of rescue trials.

TABLE 3. Rescue of SV40 from mouse kidney lines (mKS-U) transformed at various input multiplicities of irradiated SV40^a

Group	Cell line	Input multiplicity ^b		T-antigen		Avg no. of plaques formed after plating 10 ⁵ transformed cells
		PFU/cell	PFU/culture	CFU per mg of protein	IF	
I-UV	1	8	9.2×10^7	56	+	13
	2			121	+	9
	3			111	+	25
	4			100		11
	5			65		1
	6			94		42
II-UV	1	2	2.0×10^7	181	+	28
	2			103	+	5
	3			102	+	0.5
	4			162		0
	5			74		0
	6			112		2
III-UV	1	6×10^{-3}	6.9×10^4	162	+	0
	2			125	+	0.6
	3			86	+	0.2
	4			155		1
	5			117		0
	6			35		0.6
IV-UV	2	6×10^{-4}	7.8×10^3	253	+	0
	5			155	+	2
	6			76	+	0
V-UV	1	4×10^{-5}	4.8×10^2	206	+	0
	5			170	+	0
	6			188	+	0

^a Rescue trials were performed on mKS-U lines at passages 6 to 11.

^b Calculated as residual live virus.

TABLE 4. *Rescue of SV40 from clonal lines of mouse kidney cells (mKS-U) transformed at various input multiplicities of irradiated SV40^a*

Group	Cell line	Input multiplicity ^b (PFU/cell)	T antigen		Avg no. of plaques formed after plating 10 ⁵ transformed cells ^c
			CFU per mg of protein	IF	
I-UV	1	8	236	+	5(3)
	2		220	+	0(3)
	3		440	+	9(3)
	4		274	+	5(3)
	5		178	+	0(3)
	6		175	+	3(3)
II-UV	1	2	443	+	0(3)
	2		472	+	0(3)
	3		201	+	0(3)
	4		185	+	0.2; 0; 0; 0(4)
	5		224	+	0(3)
	6		250	+	0.4; 0; 0; 0(4)
III-UV	1	6×10^{-3}	112	+	0(3)
	2		378	+	0.4; 0; 0; 0(4)
	3		162	+	0(3)
	4		229	+	0(3)
	5		432	+	0.04; .04; 0; 0(4)
	6		231	+	0(3)
IV-UV	2a	6×10^{-4}	133	+	0(3)
	2b		202	+	0(3)
	5a		104	+	0(4)
	5b		107	+	0(3)
	6a		129	+	0(3)
	6b		156	+	0(3)
V-UV	1a	4×10^{-5}	106	+	0(3)
	1b		242	+	0(3)
	5a		115	+	0(3)
	5b		222	+	0(3)
	6a		114	+	0(3)
	6b		87	+	0(3)

^a Rescue trials were performed on clonal lines of mKS-U cells at passages 8 to 37.

^b Calculated as residual live virus.

^c Numbers in parentheses signify number of rescue trials.

these lines was identified as SV40 by neutralization with SV40-specific antiserum. The four positive lines have been classified as "poor" or "rare" yielders.

DISCUSSION

In the present experiments, primary mouse kidney cultures were transformed with either unirradiated or UV-irradiated SV40 at input multiplicities ranging from 0.006 to 200 PFU/cell. Rescue experiments from clonal lines transformed

by unirradiated virus demonstrated that 22 lines were "good" or "average" yielders and one was a "poor" yielder. "Good," but not "rare," yielders were obtained at the lowest input multiplicity of unirradiated virus giving transformation (0.06 PFU/cell).

In contrast, 22 of 30 clonal lines isolated from cultures transformed by SV40 irradiated to a survival of 0.01 to 0.04 were nonyielders. All of the clonal lines of groups IV-UV and V-UV (input multiplicities of 6×10^{-4} and 4×10^{-5} PFU/cell, residual live virus), and 8 of 12 lines of groups II-UV and III-UV (input multiplicities of 2 and 6×10^{-3} PFU/cell live virus) were nonyielders. However, only two of six clonal lines obtained from group I-UV (input multiplicity 8 PFU/cell live virus) were nonyielders. Presumably, defective SV40 genomes were integrated in the non-yielder lines. The positive lines of group I-UV were "good" or "average" yielders, but three of the positive lines of groups II-UV and III-UV were "rare" yielders and the fourth was a "poor" yielder. In previous studies performed at high input multiplicities, 4 of 83 lines transformed by UV-irradiated SV40, but none transformed by unirradiated virus, were "rare" yielders (7).

There are several uncertainties associated with the interpretation of these experiments. First, it is uncertain whether the input SV40 deoxyribonucleic acid (DNA) is replicated in mouse cells abortively infected with unirradiated SV40. Previous efforts to demonstrate replication were unsuccessful (10). However, the possibility that a few rounds of DNA replication occur has not been rigorously excluded. In the case of cultures infected with UV-irradiated SV40, replication of input SV40 DNA is much less likely.

Second, the number of SV40 genomes integrated into the transformed cells is uncertain. Previous studies have shown that the SV40 stocks contain about 20 particles per PFU and that about half of the PFU are adsorbed to mouse kidney cells in 2 hr at 37 C (4, 12). Thus, at an input multiplicity of 0.06 PFU of unirradiated virus per cell, 0.03 PFU/cell and 0.6 virion/cell would be adsorbed. In this case, there is a probability that 33% of the cells received one virion, 10% received two virions, and 0.000031% received seven virions. Also, about 3% of the cells probably received at least 1 PFU and 0.0004% of the cells received 3 PFU. Since the efficiency of transformation was very low [1 to 4 cells transformed among 10^7 cells (0.00002%)], selection of transformed clones from cells which received several virions cannot be ruled out. Cooperation among virions would be expected to enhance the transformation process.

Assuming that multiply infected cells were

selected, it is nevertheless not improbable that only one SV40 genome per cell was integrated. Experiments with SV40-infected 3T3 cells have indicated that only a fraction of the cells forming SV40 T antigen are transformed and that approximately 10^3 infectious units (10^5 physical particles) are required for one transformation (2). With SV40-infected human cells, 250 PFU were needed to produce one T-antigen positive cell, and about 1 in 250 T-antigen positive cells produced a transformed focus (1). It appears that double or multiple lysogens can be obtained by mixed infection of cultures with two SV40 strains at high input multiplicities (D. R. Dubbs and S. Kit, *Bacteriol. Proc.*, p. 155, 1969), but it is not known whether double transformation can also be achieved at very low input multiplicities. The tentative conclusion to be drawn is that "good" yielders may include cells with only one integrated SV40 genome. Further information on the questions raised in this discussion could, perhaps, be obtained by measuring the number of DNA equivalents per transformed cell (13).

The data presented in this study show that "rare" yielders can be expected when cells are infected at moderately low input multiplicities with UV-inactivated SV40 (0.01 to 0.04 survival). However, mostly nonyielders can be expected when the input multiplicity is very low (less than 6×10^{-4} PFU/cell residual live virus). Virus strains previously isolated from "rare" yielders transformed at high input multiplicities exhibit all known SV40 functions (7; S. Kit et al., *Bacteriol. Proc.*, p. 157, 1969). It remains to be seen whether this is also true of the virus strains from "rare" yielders isolated in the present study. The following hypotheses may account for the "rare" yielder lines and the virus strains rescued from them. (i) "Rare" yielders may contain SV40 genomes with mutations in genes essential for reversal of integration, but not for productive infection of susceptible CV-1 cells. (ii) Rescue of SV40 from "rare" yielder lines may occur through occasional recombination between defective genomes after activation of double lysogens (6). (iii) "Rare" yielder lines may contain relatively high levels of inhibitory substances which prevent activation of SV40 replication in heterokaryons of transformed and susceptible cells (1, 3).

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ADDENDUM IN PROOF

Preliminary measurements have been carried out on the percentage of mouse kidney cells that form T antigen 72 hr after infection with SV40 at input multiplicities of 24, 2.6, and 0.14 PFU per cell. These measurements indicate that about 1 in 5 to 1 in 10 of the cells that receive 1 PFU go on to synthesize T antigen.

LITERATURE CITED

1. Aaronson, S. A., and G. J. Todaro. 1968. SV40 T-antigen induction and transformation in human fibroblast cell strains. *Virology* 36:254-261.
2. Black, P. H. 1966. Transformation of mouse cell line 3T3 by SV40: dose response relationship and correlation with SV40 tumor antigen production. *Virology* 28:760-763.
3. Brailovsky, C. A., L. D. Berman, and C. Chany. 1969. Decreased interferon sensitivity and production in cells transformed by SV40 and other oncogenic agents. *Int. J. Cancer* 4:194-203.
4. Carp, R. I., S. Kit, and J. L. Melnick. 1966. The effect of ultraviolet light on the infectivity and enzyme-inducing capacity of papovavirus SV40. *Virology* 29:503-509.
5. Cassingena, R., and P. Tournier. 1968. Mise en évidence d'un "repressur" spécifique dans des cellules d'espèces différentes transformées par le virus SV40. *Compt. Rend.* 267:2251-2254.
6. Dubbs, D. R., and S. Kit. 1965. The effect of temperature on induction of deoxythymidine kinase activity by herpes simplex mutants. *Virology* 25:256-270.
7. Dubbs, D. R., and S. Kit. 1968. Isolation of defective lysogens from simian virus 40-transformed mouse kidney cultures. *J. Virol.* 2:1272-1282.
8. Dubbs, D. R., S. Kit, R. A. de Torres, and M. Anken. 1967. Virogenic properties of bromodeoxyuride-sensitive and bromodeoxyuridine-resistant simian virus 40-transformed mouse kidney cells. *J. Virol.* 1:968-979.
9. Jensen, F. C., A. J. Girardi, R. V. Gilden, and H. Koprowski. 1964. Infection of human and simian tissue cultures with Rous sarcoma virus. *Proc. Nat. Acad. Sci. U.S.A.* 52:53-59.
10. Kit, S., R. A. de Torres, D. R. Dubbs, and M. L. Salvi. 1967. Induction of cellular deoxyribonucleic acid synthesis by simian virus 40. *J. Virol.* 1:738-746.
11. Kit, S., D. R. Dubbs, P. M. Frearson, and J. L. Melnick. 1966. Enzyme induction in SV40-infected green monkey kidney cultures. *Virology* 29:69-83.
12. Kit, S., D. R. Dubbs, L. J. Piekarski, R. A. de Torres, and J. L. Melnick. 1966. Acquisition of enzyme function by mouse kidney cells abortively infected with papovavirus SV40. *Proc. Nat. Acad. Sci. U.S.A.* 56:463-470.
13. Westphal, H., and R. Dulbecco. 1968. Viral DNA in polyoma- and SV40-transformed cell lines. *Proc. Nat. Acad. Sci. U.S.A.* 59:1158-1165.