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Isolation of Defective Lysogens from Simian Virus 40-transformed Mouse Kidney Cultures

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Rescue of simian virus 40 (SV40) from hamster and murine cell lines transformed by nonirradiated or by ultraviolet (UV)-irradiated SV40 (10^{-3} to 10^{-5} survival) was studied. A combination of tests was employed to detect induction of SV40 synthesis: (i) co-cultivation with susceptible monkey kidney (CV-1) cells; (ii) treating mixtures of transformed and CV-1 cells with UV-irradiated Sendai virus (UV-Sendai) prior to co-cultivation; and (iii) plating untreated or UV-Sendai-treated mixtures of transformed and CV-1 cells with freshly trypsinized CV-1 cells. The first and second tests provided a measure of the total infectious SV40 yield per culture, and the third test provided a measure of the frequency of induction (fraction of transformed cells giving rise to infectious centers). With the combination of tests, SV40 was rescued in all trials from TSV-5 hamster cells, mKS-BU100 mouse cells, and from several lines of mouse kidney cells transformed by UV-irradiated SV40 (mKS-U lines). The frequency of induction was about 7×10^{-2} for TSV-5 cells, about 3×10^{-3} for mKS-BU100 cells, greater than 10^{-4} for the mKS-U lines which were "good" yielders, and about 10^{-5} to 10^{-4} for the mKS-U lines which were "average" yielders. SV40 of a plaque type different from parental virus was rescued from four of the mKS-U cell lines. Virus was also easily rescued from: (i) tumor cells produced from the mKS-A line of transformed mouse kidney cells; (ii) mouse kidney cells transformed by SV40 which had been rescued from mKS-BU100 cells; and (iii) tumor cells (HATS) which had been produced by inoculating newborn hamsters with SV40 rescued from mKS-BU100 cells. The frequency of induction of HATS cells was of the same order of magnitude as the frequency of induction of TSV-5 cells. In a study of the kinetics of virus induction, it was shown that SV40 could be detected 28, 40, and 48.5 hr after UV-Sendai treatment of mixtures of CV-1 and TSV-5, HATS, or mKS-BU100 cells, respectively. Although all of the mKS-U lines contained the SV40-specific tumor antigen, some were *poor* virus yielders (SV40 was recovered in less than 50% of the trials) and five lines were *rare* virus yielders (SV40 recovered only once in four or more trials). Forty-eight mKS-U lines were nonyielders; SV40 was never recovered by any test used thus far. UV-Sendai-treated mixtures of pairs of nonyielder mKS-U lines with CV-1 cells also did not yield infectious virus. Various factors affecting rescue have been discussed. The mKS-U lines which were poor virus yielders, rare yielders, or which never yielded virus have been classified tentatively as "defective lysogens" which contain mutational lesions at loci essential for detachment of SV40 from integration sites or for SV40 replication, or for both.

A number of simian virus 40 (SV40)-transformed cell lines which do not spontaneously produce virus can be induced to do so when cocultivated with susceptible green monkey kidney cells. Treatment of mixtures of transformed and susceptible cells with ultraviolet (UV)-irradiated Sendai virus (UV-Sendai) facilitates the activation of infectious SV40 synthesis, probably by increasing cell fusion and heterokaryon formation. Cloning experiments and characterization of SV40 rescued from these transformed cell lines have shown that the entire complement of SV40 genetic information is maintained in every transformed cell (3, 4, 7, 9, 10, 15, 18, 22, 23).

In contrast, other SV40-transformed cell

lines have thus far not yielded infectious virus although they contain the SV40-specific tumor (T) antigen (3, 8, 15, 19, 22). Several hypotheses can be invoked to explain the failure of these cell lines to yield infectious virus: (i) only a fragment of the SV40 genome is integrated in these cells; (ii) the incidence of fusion with susceptible cells is extremely low; or (iii) the complement of viral or cellular genes contains mutations which block the reversal of the integrated state or virus replication, or both.

In connection with the third hypothesis, it seemed likely that transformed cell lines with mutant SV40 genes could be obtained by transforming mouse kidney cells with SV40 which had been damaged by UV irradiation (6). These "defective lysogens" might be useful for studying the detailed mechanisms of SV40 rescue and could provide a source for viral mutants (5). Isolation of cell lines transformed by UV-irradiated SV40 was therefore undertaken. A preliminary report on the isolation and characteristics of cell lines transformed by UV-irradiated SV40 has already been presented (D. R. Dubbs and S. Kit, Bacteriol. Proc., p. 177, 1968).

MATERIALS AND METHODS

Cell lines. CV-1 cells, an established line of green monkey kidney cells (11), were used as indicator cells and were grown in monolayer cultures as previously described (12). The origins of the SV40-transformed mouse and hamster cell lines used in this study are shown in Table 1. The transformed cells were grown in monolayer culture and were subcultured twice weekly. The mKS-BU100 cells were grown routinely in medium containing either 25 or 100 μg of 5-bromodeoxyuridine per ml. Transformed cell lines were cloned without a feeder layer in medium containing 1% SV40 antisera as previously described (7).

All transformed cell lines used in this study contained SV40 T antigen as determined by complement fixation (*CF*). None of the transformed cell lines spontaneously released SV40. Cell-free extracts prepared from cultures of each transformed cell line failed to yield infectious SV40 when assayed on CV-1 monolayers.

Virus strains. SV40 clone 307L was propagated and assayed in monolayer cultures of CV-1 as previously described (12). Sendai virus, obtained from H. Koprowski, Wistar Institute, Philadelphia, Pa., was propagated in the allantoic cavity of 10- to 11-day-old developing chick embryos. The allantoic fluid was harvested 48 to 72 hr postinoculation and was clarified by low-speed centrifugation. The virus particles were pelleted by centrifugation (Spinco model L-2, no. 30 rotor) at 30,000 rev/min for 30 min and were concentrated 10-fold in phosphate-buffered saline (PBS). Hemagglutination tests employed equal volumes of serially diluted virus and 4% chicken erythrocytes at 4 C. One hemagglutinating unit (HAU) is defined as the smallest amount of virus which produced complete hemagglutination. Sendai virus was inactivated by UV irradiation for 5 min at a distance of 25 cm from a Sylvania G30T8 germicidal lamp ($\sim$70 ergs per mm^2 per sec at 253.7 nm); the UV irradiation reduced the infectivity of Sendai virus from $>10^{10.5}$ to $<10^{2.5}$ ID_{50} per ml for embryonated eggs.

Antiserum. SV40 antiviral sera prepared either in horses or in baboons and SV40 antitumor sera from hamsters bearing SV40 transplant (virus-free) tumors were obtained from Flow Laboratories, Inc., Rockville, Md. Neutralization tests were performed as previously described (7).

CF tests. Preparation of cell extracts (7) and demonstration of SV40 T antigen have been described previously (14). For comparative purposes, the antigen titers are expressed as CF units per milligram of protein.

Treatment of cell mixtures with UV-irradiated Sendai virus. The procedure used for fusing transformed and CV-1 cells with UV-Sendai virus is shown in Fig. 1. Mixtures of transformed cells and CV-1 cells in a ratio of 1:1 or 1:2 were suspended in PBS containing UV-Sendai (irradiated for 5 min). Standard conditions employed 8,000 HAU of UV-Sendai in 1 ml for each 5×10^6 transformed cells. Control cell mixtures were suspended in 1 ml of PBS without UV-Sendai. The mixtures were chilled in an ice bath for 10 min to permit agglutination of the cells and then were transferred to a reciprocal shaker-incubator for 20 min at 38 C. The cells were washed with growth medium to remove UV-Sendai and were resuspended in growth medium.

For determination of SV40 yield, samples of control or UV-Sendai-treated mixtures were seeded in 8-oz (0.236 liter) prescription bottles in 20 ml of medium. Initially, 2×10^6 total cells were seeded per bottle. However, in later experiments, 3×10^6, 4×10^6, or 6×10^6 total cells were used in an attempt to increase the probability of detecting SV40 in transformed cell strains which had not previously yielded SV40. The cultures were incubated at 37 C for 7 days; then the cells were scraped from the glass into the growth medium and were disrupted by sonic oscillation at 4 C and 10 kc for 1 min. In later experiments, the cells were resuspended in one-tenth the initial volume of supernatant fluid prior to sonic treatment. SV40 was assayed by plaque formation on CV-1 monolayers (12).

To determine the frequency of SV40 induction in control or UV-Sendai-treated mixtures of transformed and CV-1 cells, the cells were diluted serially and were plated in dishes (60 mm) along with an extra 10^6 freshly trypsinized CV-1 cells (Fig. 1). The dishes were incubated overnight to permit cell attachment; then the fluid medium was removed and an agar overlay (5 ml) consisting of R5a medium (12) with 7.5% fetal calf serum and 1% agar was added. After 10 days of incubation, a second agar overlay (5 ml) consisting of R5a medium supplemented with 0.5% lactalbumin hydrolysate, 0.1% Yeastolate (Difco), 1% fetal calf serum, and 1% agar containing neutral red was added. Plaques were counted between 14 and 25 days after plating. In some experiments, un-

TABLE 1. *SV 40-transformed cell lines*

Cell line	Animal species and tissue	Comments	Reference
mKS-A	Mouse kidney	Transformed in vitro by SV40 clone 307L	(7)
mKS-B			
mKS-A clone 4	Mouse kidney	Clonal isolates from mKS-A	(7)
mKS-A (TU-1)	Mouse tumor	Tumor produced in BALB/c mice after inoculation of mKS-A cells	
mKS-BU100	Mouse kidney	dBU resistant strain of mKS-B	(7)
mKS-ST2	Mouse kidney	Transformed in vitro by SV40 recovered from mKS-BU100 cells	
mKS-U	Mouse kidney	Transformed in vitro by UV-irradiated SV40	
HATS	Hamster tumor	Tumor induced by SV40 recovered from mKS-BU100 cells	
TSV-5 clone 2	Hamster tumor	Tumor induced by SV40	(22)
H-50	Hamster tumor	Tumor induced by SV40	(17)
2X-10	Hamster kidney	Transformed in vitro by SV40	(17)

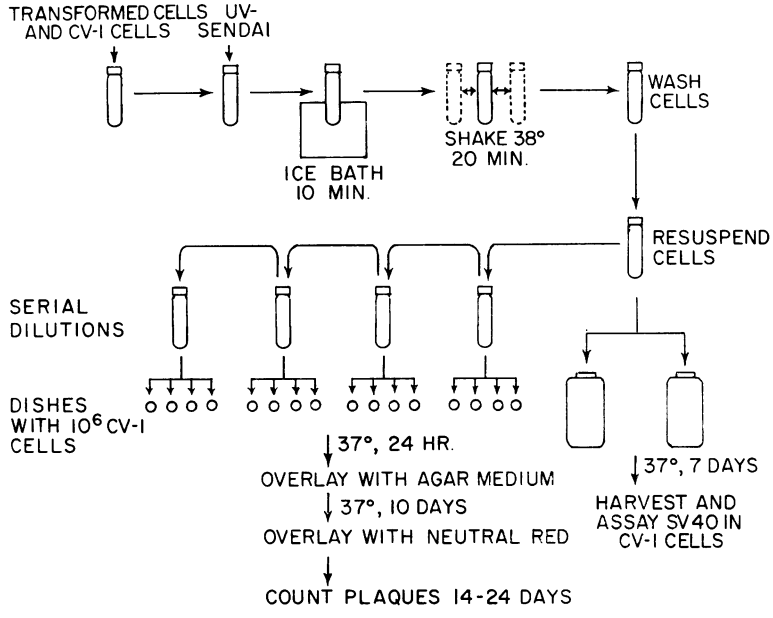

FIG. 1. *Procedure for fusing transformed and CV-1 cells with UV-irradiated Sendai virus.*

treated transformed cells were mixed with CV-1 cells and were plated directly in dishes without being first subjected to the chilling and shaking steps.

Direct assay of cell extracts. Four- or five-day-old cultures containing 10×10^6 to 30×10^6 cells were used. Cells were scraped from the bottle into the supernatant fluid, sedimented by low-speed centrifugation, and resuspended in one-fifth the original volume of supernatant fluid. The cells were disrupted by sonic treatment and were assayed for SV40 on CV-1 monolayers.

Definitions. Virus yielders refers to cell lines from which SV40 can be rescued by co-cultivation with CV-1 cells with or without UV-Sendai treatment. *Non-virus yielders* refers to cell lines from which infectious SV40 has not yet been recovered by (i) co-cultivation with CV-1 cells, (ii) treatment of mixtures of transformed and CV-1 cells with UV-Sendai, or (iii) production of plaques when untreated transformed or UV-Sendai-treated mixtures of transformed and CV-1 cells were plated with CV-1 cells.

Frequency of induction refers to the fraction of transformed cells (either untreated or after fusion with CV-1 cells in the presence of UV-Sendai) which gives rise to infectious centers when plated with freshly trypsinized CV-1 cells and overlaid 24 hr later with agar medium (Fig. 1).

RESULTS

Evaluation of testing methods. A series of initial experiments were performed to determine the most suitable conditions for rescuing SV40

from transformed cell lines. In a previous study (7), various lines of mouse kidney cells transformed by SV40 were co-cultivated with susceptible CV-1 cells without UV-Sendai treatment Except for clone 4 of the mKS-A line, all of the parental and clonal lines yielded SV40. At the time of that study, mKS-A clone 4 failed to yield SV40 in seven trials. In a total of 13 trials in which mKS-A clone 4 has now been tested by cocultivation without UV-Sendai treatment, 2 trials yielded SV40; in each case, a single plaque appeared quite late on the titration plates. The mKS-A clone 4 cells were then recloned and seven subclones were isolated and tested 10 to 12 times by cocultivation for induction of SV40 formation. The incidence of successful trials varied from 0 out of 11 with mKS-A clone 4-2 to 4 out of 10 with mKS-A clone 4-8 (Table 2, third column). In nearly all instances when virus was detected, only one or two plaques were found on titration plates, indicating that only about 100 to 200 plaque-forming units (PFU) per mixed culture were produced in 5 to 7 days.

In some cases, recovery of SV40 can be obtained after UV-Sendai treatment of mixtures of transformed and susceptible cells, even though rescue is unsuccessful without UV-Sendai treatment (9, 15, 23). Seven secondary clones of mKS-A clone 4 were, therefore, tested for SV40 induction by treating mixtures of transformed and CV-1 cells with UV-Sendai. Four of the clonal lines which failed to yield SV40 in control mixtures did yield SV40 after UV-Sendai treatment, and two clonal lines (4-1 and 4-3) which were weakly positive by cocultivation gave enhanced yields of SV40 (Table 2). However, clone 4-7 failed to yield SV40 in two trials and clones 4-2 and 4-6 each yielded SV40 in only one of two trials in spite of UV-Sendai treatment.

The secondary clones were also tested by plating transformed cells directly in petri dishes along with 10^6 freshly trypsinized CV-1 cells (Table 2). Plaques were produced in every instance; they were picked from dishes receiving 5 of the 7 clonal lines and were shown to be SV40. Therefore, we decided to use a combination of tests in screening transformed cell lines. Furthermore, to increase the sensitivity of the methods, cell harvests in some experiments were concentrated 10-fold prior to determining the SV40 yield.

UV-Sendai treatment and frequency of induction. The "frequency of induction" is a useful parameter in characterizing transformed cell lines. To

TABLE 2. *Isolation of SV40 from secondary clonal lines of mKS-A clone 4*

Clonal line of mKS-A	T-antigen (CFU[a] per mg of protein)	Trials positive by cocultivation (no UV-Sendai treatment)	Avg SV40 yields (PFU) per mixed culture[b]		Avg no. of plaques formed after plating indicated no. of transformed cells[c]	
			Control	UV-Sendai	10^6	10^5
		%				
Clone 4-1	74	20	5×10^1	2×10^2 1.6×10^3	1	3
Clone 4-2[d]	533	0	0	0 6.6×10^3	2	7.2
Clone 4-3	106	8	1×10^2	2×10^3 1.1×10^3	2	1.8
Clone 4-6[d]	142	10	0	1×10^2 0	1.2	0.8
Clone 4-7[d]	152	9	0	0	0.5	0
Clone 4-8[d]	188	40	0	2.5×10^2 6.6×10^2	4.2	5.0
Clone 4-10[d]	355	18	0	7.0×10^2 1.6×10^2	1.2	4.8

[a] Complement fixation units.

[b] CV-1 (5×10^6) and 5×10^6 transformed cells were resuspended either in 1 ml of UV-irradiated Sendai virus (5,000 to 8,000 HAU) or in 1 ml of PBS. Bottle cultures were inoculated with 2×10^6 mixed cells and were harvested 7 days after plating.

[c] Serial dilutions of the transformed cells were prepared, and 1 ml (containing indicated number of cells) was plated in petri dishes (60 mm) containing 10^6 freshly trypsinized CV-1 cells in 4 ml of growth medium. Agar medium was added 24 hr after plating. A second agar overlay containing neutral red was added at 10 days, and plaques were counted at 15 to 21 days.

[d] SV40 has been isolated from plaques appearing after plating transformed cells with CV-1 cells. No plaques were picked from clones 4-1 or 4-3.

determine the most suitable conditions for measuring the frequency of induction, the effect of UV-Sendai treatment on the fusion of mKS-BU 100 with CV-1 cells was studied. The results indicated that the maximal number of virus yielders in cell mixtures was obtained when 8,000 HAU of Sendai virus (UV-irradiated for 3 to 7 min) was used for 5×10^6 transformed mKS-BU 100 cells. Higher induction frequencies were also obtained when the ratio of transformed cells to CV-1 cells was increased from 1:1 to 1:2. However, in control mixtures treated with PBS rather than with UV-Sendai, changing the ratio of mKS-BU 100 to CV-1 cells did not enhance the frequency of induction of SV40.

Rescue of SV40 from various transformed cell lines. A number of transformed cell lines available in the laboratory were tested for virus yield per culture and for frequency of induction of SV40 (Table 3). The cell lines differed considerably in these properties. When UV-Sendai-treated mixtures of CV-1 and TSV-5 hamster cells were plated with CV-1 cells, about 7% of the transformed cells produced plaques. In contrast, about 0.3% of the mKS-BU 100 mouse cells in UV-Sendai-treated mixtures and about 0.04% of the mKS-A (TU-1) cells produced infectious centers, but no infectious centers were obtained with the H-50 and 2X-10 lines of transformed hamster cells.

Kinetics of SV40 induction in mixed cultures of transformed and indicator cells. In mixtures of TSV-5 and CV-1 cells treated with UV-Sendai, infectious SV40 was detected as early as 28 hr after fusion and then rapidly increased until 54 hr (Fig. 2). Thus, SV40 replication is detected almost as early in UV-Sendai-treated mixtures of TSV-5 and CV-1 cells as in CV-1 cells after productive SV40 infection (24 hr). In mixtures of TSV-5 and CV-1 cells not treated with UV-Sendai, infectious SV40 was not detected as early as in UV-Sendai-treated mixtures. However, by 44 hr after mixing, SV40 titers were nearly equal in the two types of cultures.

Infectious SV40 was not present in mixed cultures of CV-1 and tumor cells (HATS) before 40 hr after plating, regardless of whether the mixtures were treated with UV-Sendai or not (Fig. 2). Infectious virus titers then increased rapidly between 48 and 96 hr, but increased faster in UV-Sendai-treated cultures than in control cultures. In similar experiments, SV40 was detected in UV-Sendai-treated mixtures of mKS-BU 100 and CV-1 cells 48.5 hr after mixing (13).

Successive transformation by rescued SV40. In a previous study, it was shown that SV40 recovered from transformed mouse kidney cells expressed the same viral functions as parental SV40 in either productively infected CV-1 cells or in abortively infected mouse kidney cells and was able to transform mouse kidney cells (7). Tournier et al. (22) have also shown that SV40 recovered from TSV-5 cells was able to transform primary embryonic hamster cells and was oncogenic for newborn hamsters. However, the cell strains which they established from the hamster tumor or transformed cells failed to yield SV40 when cocultivated with monkey kidney (BSC-1) cells. Therefore, it was of interest to learn whether the entire SV40 genome was successfully integrated and released during successive transformation, or whether part of the SV40 genome was lost. To answer this question, individual cultures of mouse kidney cells were transformed by SV40 rescued from mKS-BU 100 cells. Nine transformed lines were obtained from cultures infected with rescued SV40 at an input multiplicity of 110 PFU/cell. All contained the SV40 T antigen, but cell-free extracts did not yield SV40.

TABLE 3. *Frequency of induction of SV40 and virus yields in mixed cultures of transformed mouse or hamster cells and CV-1 cells*[a]

Transformed line	T-antigen (CFU per mg of protein)	Avg no. of plaques formed after plating indicated no. of transformed cells				Avg SV40 yield (PFU) per mixed culture at 7 days
		10^2	10^3	10^4	10^5	
TSV-5	62	7	TMC[b]	TMC		2.8×10^6
HATS	342		18	TMC		4.3×10^7
mKS-BU100	312		3	24	TMC	1.7×10^6
mKS-A (TU-1)	188			4	43	8.4×10^4
H-50	286			0	0	0
2X-10	162			0	0	0

[a] Mixtures of transformed and CV-1 cells were treated with UV-Sendai before plating (Fig. 1).
[b] Too many to count.

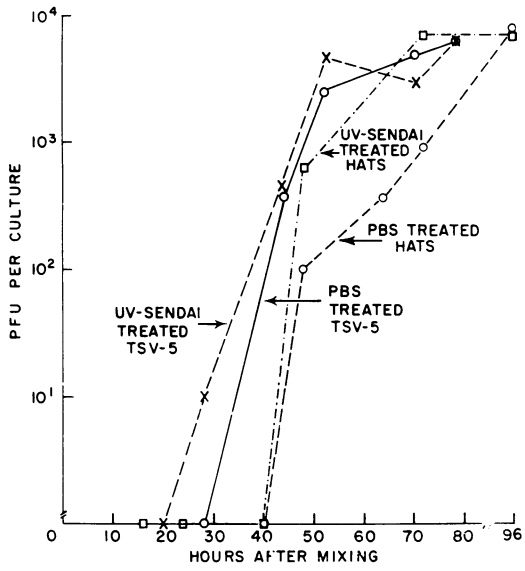

FIG. 2. *Kinetics of recovery of SV40 virus from mixtures of transformed (TSV-5 or HATS) and susceptible (CV-1) cells. Transformed cells and CV-1 cells were mixed at a ratio of 1 : 2 and were resuspended in either PBS or UV-Sendai virus (8,000 HAU per 5 million transformed cells). The cell mixtures were chilled at 4 C for 10 min and were shaken at 38 C for 20 min. Cultures were inoculated with 10^6 transformed and 2 × 10^6 CV-1 cells in 20 ml of growth medium. At indicated times, cells were harvested from two cultures, pooled, and resuspended in 4 ml of supernatant fluid. Cells were disrupted by freezing and thawing and sonic treatment prior to assay on CV-1 monolayers for SV40.*

However, all nine strains yielded SV40 after fusion with CV-1 cells in the presence of UV-Sendai.

Three clonal lines were isolated and tested for SV40 induction between passages 10 and 18. SV40 was recovered from all three lines, although one clonal line was a much better yielder than the others.

SV40 rescued from mKS-BU 100 cells was also used to induce tumors in newborn hamsters. Of 12 hamsters inoculated with 1.5 × 10^6 PFU of virus, 10 developed tumors in 3 months. One of the tumors was removed, trypsinized, and used to establish a cell line (HATS, Table 1). The HATS cells yielded virus readily in UV-Sendai-treated mixtures with CV-1 cells (Table 3); the frequency of induction was approximately 1.8 × 10^{-2}. A clonal line isolated from HATS cells also had an induction frequency greater than 10^{-3}. These studies indicated that infectious virus can be recovered after two successive transformation cycles. However, the frequency of induction of SV40 may vary considerably in clonal lines obtained after successive transformation.

Transformation by UV-irradiated SV40. UV-irradiated polyoma has been shown (1, 2, 16) to transform cells in vitro. In an effort to isolate defective lysogens, it was decided to first damage the SV40 genome and then to use the damaged virus for transforming mouse kidney cells.

The kinetics of UV-inactivation of SV40 are shown in Fig. 3. After 5 min of UV, the infectivity titer was decreased by approximately 3 logs; after 10 min of UV, by 4.5 logs; and after 15 min of UV, by 5 logs. One group of 7-day-old primary mouse kidney cultures containing 5 × 10^6 cells per bottle was infected with nonirradiated SV40 at an input multiplicity of either 186 or 30 PFU per cell, and other groups of replicate cultures were infected with the same volume of SV40 irradiated for 5, 10, and 15 min, respectively. For example, in one of the experiments, cultures infected with SV40 irradiated for 5 min

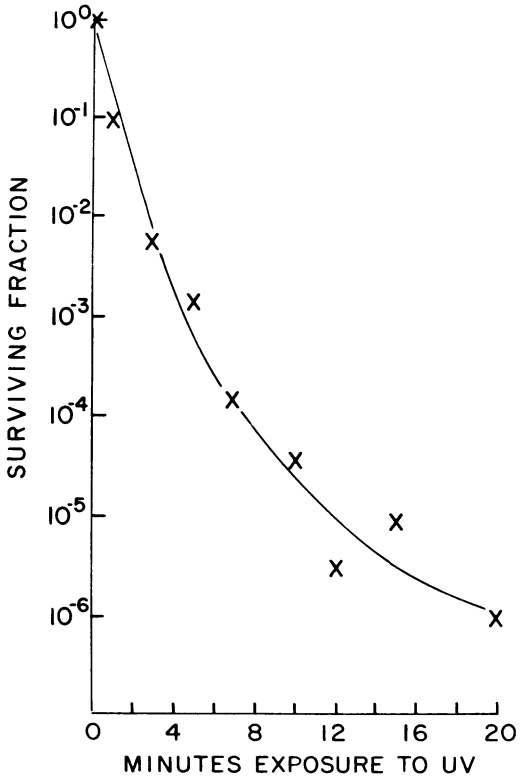

FIG. 3. *Inactivation of SV40 by UV light. Culture dishes (60 mm) containing 1.6-ml samples of SV40 clone 307L were placed 17.5 cm away from a Sylvania G30T8 germicidal lamp (~70 ergs per mm² per sec at 253.7 nm).*

received 0.35 live PFU per cell, those infected with SV40 irradiated for 10 min received 0.098 live PFU per cell, and those infected with SV40 irradiated for 15 min received 0.002 live PFU per cell. In each group, one half of the cultures were trypsinized and each was passaged to two bottles on day 2. The remainder was subcultured on day 6. The cultures were fed twice weekly. Transformed colonies first appeared at about 21 days postinoculation. In general, the cultures infected with SV40 irradiated for 5 min contained more colonies of transformed cells than did those infected with unirradiated virus. Cultures infected with SV40 irradiated for 15 min contained only one to five colonies. Each transformed culture was used to establish a cell line, which was cloned after three to five passages. In three separate experiments, a total of 83 clonal isolates, each from an individual transformed culture, was established (mKS-U1 to mKS-U102). Each of the 83 cell lines contained SV40 T antigen.

Rescue of SV40 from cell lines transformed by UV-irradiated virus. The mKS-U lines were tested for induction of SV40 by (i) cocultivation with CV-1 cells; (ii) by treatment of cell mixtures with UV-Sendai; and (iii) by formation of infectious centers after plating untreated or UV-Sendai-treated mixtures with CV-1 cells. Except where indicated in Table 4, all mKS-U cell lines were tested at least four times with a combination of one or more of the above methods.

The mKS-U cell lines were classified into five groups: nonyielders, rare yielders, poor, average, and good yielders (Table 4). Of the 83 mKS-U lines, 48 failed to yield SV40 in any test to date. Five mKS-U lines yielded virus on at least one occasion, but only in small amounts. These five lines have been classified as *rare* yielders. For example, a total of 2.6×10^2 PFU per culture was recovered from mKS-U18, passage 9. Five plaques were picked and all were identified as containing SV40. However, the next six trials with the mKS-U18 line were negative. Virus was also recovered from a mixed culture of mKS-U16, passage 19, and CV-1 cells treated with UV-Sendai. The virus yield was 9.3×10^3 PFU per culture, but five subsequent trials were negative.

From a number of the mKS-U lines, SV40 was recovered in 50% or less of the tests, always in low yields per culture. These lines were classified as *poor* yielders. In the case of the mKS-U1 and mKS-U3 lines, the infectious centers produced after plating cell mixtures treated with UV-Sendai were very small and indistinct. Similarly, virus yields from mixed cultures of mKS-U1 or mKS-U3 and CV-1 cells were of the small, indistinct plaque type which required approximately 7 days longer than usual to develop to a countable size. No wild-type plaques were obtained from mKS-U3. However, large wild-type plaques occasionally appeared from virus rescued from mKS-U1. When these wild-type plaques were picked and propagated in CV-1 cells, progeny virus also gave wild-type plaques which developed at a normal rate.

The remaining cell lines yielded SV40 every time they were tested and were classified as either *good* or *average* yielders. The frequency of induction of good yielders was greater than 10^{-4} and that of average yielders was about 10^{-5} (Table 4). SV40 isolated from most of the good and average yielders was similar to parental SV40 in plaque morphology. However, mKS-U4 always produced infectious centers which were large and had fuzzy edges. SV40 isolated from mixed cultures of mKS-U4 and CV-1 cells also produced this plaque morphology, and the plaques appeared at approximately the same time as did wild-type plaques. Progeny virus from mKS-U4 infectious centers never yielded SV40 with wild-type plaque morphology. The mKS-U88 line produced infectious centers of small plaques with distinct boundaries. Virus rescued from mKS-U88 likewise produced small but distinct plaques which developed more slowly than the wild type but faster than SV40 rescued from mKS-U3. No wild-type plaques were produced.

Other properties of the viruses isolated from mKS-U lines of transformed cells have not been scrutinized as yet; however, 15 strains of SV40, including all of those with altered plaque morphology, were neutralized by anti-SV40 sera. Virus was not recovered from cell-free extracts prepared by sonic disruption of the transformed cells in the absence of CV-1 cells.

The relationship between the percentage of cell strains which yield SV40 and the duration of the UV-irradiation of the input virus is shown in Table 5. In previous experiments, when unirradiated SV40 was used to transform mouse kidney, all of the resulting cell strains were virus yielders (7). In contrast, when the SV40 was irradiated for 5 min, only 47% of the cell lines were virus yielders. When the irradiation time was extended to 10 or 15 min, 33 and 25%, respectively, of the cell lines were yielders.

Triple mixtures. To learn whether complementation could occur between pairs of defective lysogens, the mKS-U cells were fused with CV-1 cells in triple mixtures. We used 14 mKS-U lines. These included a poor virus yielder (mKS-U1), 2 lines from which SV40 had been recovered

TABLE 4. *Isolation of infectious SV40 from mouse kidney cells transformed by UV-irradiated SV40*[a]

Classification[b]	Strain	T-antigen (CFU per mg of protein)	Input multiplicity[c] (PFU per cell)		Avg no. of plaques formed after plating indicated no. of UV-Sendai-treated transformed cells				Virus yield per UV-Sendai mixed culture (PFU)
			Total	Live	10^6	10^5	10^4	10^3	
Good	mKS-U8	134	186	0.35		17	2	0.3	5.6×10^5
	mKS-U10	258	186	0.35		36	5		$>1.0 \times 10^6$
	mKS-U13	98	186	0.098			19	3.0	1.2×10^7
	mKS-U24	145	186	0.098		61	9	2.0	7.2×10^5
	mKS-U70	(1:16)[d]	30	0.016		20			1.6×10^4
	mKS-U75	182	30	0.016		19			$>1.0 \times 10^4$
	mKS-U87	194	30	0.016		>50			$>1.0 \times 10^4$
	mKS-U88[e]	82	30	0.016		33			7.9×10^3
Average	mKS-U4[f]	180	186	0.35		3.3	0.3	0	3.4×10^4
	mKS-U6	57	186	0.35		4.5	0.3		2.2×10^3
	mKS-U42	125	30	0.006		2.8			2.6×10^5
	mKS-U59	298	30	0.016	11	3.2			1.5×10^3
	mKS-U64	(1:64)	30	0.016	42	7.2			$>1.0 \times 10^4$
	mKS-U71	156	30	0.016	14	2.2			
	mKS-U73	111	30	0.016	5	1.6			3.1×10^4
	mKS-U77	192	30	0.016	13	2.8			2.8×10^3
	mKS-U83	216	30	0.016	15	3.8			5.2×10^3
	mKS-U93	91	30	0.016	2	1.8			$>1.0 \times 10^4$
	mKS-U96	193	30	0.016		3.8			4.6×10^3
Poor	mKS-U1[g]	256	186	0.35	0	0.5	0		3.0×10^2
	mKS-U3[g]	501	186	0.35	0	0.5			8.0×10^2
	mKS-U46	54	30	0.016	0	0.6			0
	mKS-U61	(1:8)	30	0.016	0	0			8.8×10^3
	mKS-U62	108	30	0.016	1.8	0			
	mKS-U65	235	30	0.016	0	0			1.2×10^2
	mKS-U79	254	30	0.016	0	0.6			1.8×10^4
	mKS-U80	148	30	0.016	0.6	0			0
	mKS-U82	170	30	0.016	0.2	0			0
	mKS-U94	241	30	0.016	0.2	0			0
	mKS-U102	(1:8)	30	0.016	0	0			2.0×10^1

[a] All mKS-U lines listed above were transformed by SV40 irradiated for 5 min, except for mKS-U13 and mKS-U14 which were irradiated for 10 min and mKS-U42 which was irradiated for 15 min.

[b] In addition to the mKS-U lines shown above, the following lines were classified as *rare* virus yielders: mKS-U12, -U16, -U18, -U23, and -U31. Virus was recovered on at least one occasion in extremely small yields. Virus has not yet been recovered in four or more trials from lines mKS-U2, -U5, -U7, -U9, -U11, -U14, -U17, -U19, -U20, -U21, -U22, -U25, -U26, -U27, -U28, -U29, -U30, -U32, -U32A, -U36, -U41, -U43. An additional 26 lines tested only once failed to yield SV40.

[c] Total input multiplicity equals number of PFU per cell measured before irradiation. Live input multiplicity equals number of surviving PFU per cell measured after irradiation.

[d] Numbers in parentheses equal CF titer (highest dilution of antigen giving 3^+ or 4^+ fixation in the presence of 4 units of antibody).

[e] Plaques are very small but distinct.

[f] Plaques are large with fuzzy edges.

[g] Plaques are very small and indistinct. Positive experiments are cited. Virus was recovered in about 50% of tests.

on only one occasion (mKS-U18 and mKS-U31), and 11 lines from which SV40 had never been recovered. Five million cells from each of two mKS-U lines were mixed either with 5×10^6 CV-1 cells (one experiment) or with 10^7 CV-1 cells (two experiments) and were treated with 16,000 HAU of UV-Sendai in 2 ml. The mixed UV-Sendai-treated cells were planted in bottle cultures and, in two of the three experiments, were also plated with CV-1 cells as for determining induction frequencies. Each of the 14 cell lines was tested in mixtures with 3 or 4 other

TABLE 5. *Relationship between the duration of UV exposure of input SV40 and the percentage of transformed lines which are virus yielders*

Duration of UV irradiation of SV40	Total cell lines isolated	No. of lines which are		Per cent of lines which are virus yielders
		Virus yielders	Non-yielders	
min				
0	11[a]	11	0	100
5	62	29	33	47
10	9	3	6	33
15	12	3	9	25

[a] Results of a previous study (7).

mKS-U lines. Virus was recovered from only one mixture in the three experiments; this mixture contained mKS-U1, a poor virus yielder, as one member of the pair of mKS-U lines.

DISCUSSION

Either unirradiated or UV-irradiated SV40 can induce tumors in newborn hamsters and transform cells in culture. Indeed, Defendi and Jensen (6) have shown that the incidence of tumors with 10^7 PFU of SV40 irradiated to 10^{-3} survival is greater than that with unirradiated virus. In this study, transformation of primary mouse kidney cells appeared to be more efficient with SV40 irradiated to 10^{-3} survival than with unirradiated SV40. However, since all of the cultures received a considerable amount of fully infectious SV40 (10^4 to 10^6 PFU/culture), it is possible that the transformation events were in some instances actually caused by infectious SV40 *survivors*. Some of the mKS-U lines classified as good or average yielders probably fall into this category. Three lines of evidence, however, suggest that UV-damaged SV40 was responsible for transformation of at least some of the 83 mKS-U lines. (i) In a previous study involving 11 lines of mouse kidney cells transformed by parental SV40, virus was recovered from all 11 lines by cocultivation with CV-1 cells, whereas in this study only 42% of the lines were virus yielders. (ii) The per cent of lines yielding SV40 was lower when SV40 irradiated for 15 min was used to transform mouse kidney cells (25%) than when SV40 irradiated for 5 min was used (47%). (iii) Of the 30 lines which regularly produced SV40 in mixed culture, 4 lines yielded SV40 with plaque morphology different from the parental SV40.

It has been estimated that SV40-transformed cell lines contain 10 to 60 equivalents of deoxyribonucleic acid (DNA) of SV40 (24). The DNA is integrated in transformed cell nuclei in a conformation which is noninfectious (13; 23). It is unclear, however, how many different types of genomes are integrated in a given cell and whether they are integrated in tandem (catenates) or at independent sites. The present rescue experiments have shown that the mKS-U3, mKS-U4, and mKS-U88 cells always yield SV40 of a distinctive plaque type. However, the mKS-U1 line occasionally yields two plaque types; this suggests either that dissimilar SV40 genomes may be integrated, or that the molecular events leading to detachment of a complement of SV40 genetic information from an integrated site may involve genetic recombination. The first possibility is supported by the finding that hamster tumor cells may be doubly transformed by both SV40 and polyoma virus (21), and raises the question of whether SV40 genomes may be detached from different integrated sites with equal facility.

Activation of SV40 DNA and virus synthesis is readily accomplished in UV-Sendai-treated mixtures of certain transformed and monkey kidney lines. With the TSV-5 cells (22), plaques are produced when cell mixtures (with or without UV-Sendai treatment) containing 100 TSV-5 cells are plated with CV-1 cells. The induction frequency for HATS cells exceeded 10^{-2} and that of mKS-BU100 cells varied from 10^{-3} to 10^{-2}. Formation of infectious DNA has been detected 19 hr after fusion of TSV-5 or mKS-BU100 cells with CV-1 cells (13); in the case of TSV-5 cells, infectious virus has been detected 28 hr after fusion. These events occur within the same time span as a single cycle of productive infection of CV-1 cells by SV40. However, the frequency of induction, the incidence of successful rescues, the total yield of SV40 per culture, and the kinetics of virus synthesis can be considerably lower in many transformed cell lines than with TSV-5 or HATS cells.

It is likely that a number of factors, in addition to those already discussed, contribute to this result. Rescue probably depends on a combination of properties of the SV40 genome, the transformed cell in which the viral genome is integrated, and the permissive monkey cell line employed. Preliminary experiments from this laboratory suggest that the incidence of fusion of transformed lines with CV-1 cells is not uniform. Transformed lines which fuse very poorly with susceptible monkey cells may not develop infectious virus, even though viral genes concerned with detachment from the integrated state and replication are intact. On the other hand, mutations in genes essential for detachment or virus

replication, or both, would prevent rescue of virus even when more than one "headful" of DNA was present in the transformed cell. In this connection, it is of interest that H-50 hamster cells apparently contain about 60 equivalents of SV40 DNA (24) but fail to synthesize either infectious DNA or virus after UV-Sendai treatment of cell mixtures (13). The failure to rescue SV40 from the nonyielder lines of mKS-U cells may also be ascribed, in part, to mutations in the viral DNA. The poor yielders and the rare yielders could include leaky mutants, recombinants, or, perhaps, revertants to pseudowild type.

To obtain SV40 rescue through recombination or complementation of viral genomes, mixtures of pairs of nonyielder mKS-U lines and CV-1 cells were made. However, these triple mixtures have not resulted in SV40 rescue to date. This could be due to a number of things: (i) the cell types employed belonged to the same complementation group, or (ii) *nuclear* fusion might be essential for successful recombination. It is known that in heterokaryons all nuclei may acquire SV40 gene products, such as the T antigen, in the absence of DNA synthesis (20). The fact that "virus-specific" changes occur in BSC-1 nuclei of heterokaryons does not necessarily signify, however, that viral DNA is transmitted to or replicates in the heterologous nuclei of heterokaryons (23). Assuming that complementation does lead to the formation of virus particles, harvests of the defective viruses would be expected to yield plaques only when indicator cells were simultaneously infected with both of the complementing mutants. In the event that two or more dissimilar SV40 genomes are integrated in a single transformed cell, occasional recombination from a mating pool could occur.

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