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Demonstration of Infectious Deoxyribonucleic Acid in Transformed Cells

I. Recovery of Simian Virus 40 from Yielder and Nonyielder Transformed Cells

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Deoxyribonucleic acid (DNA) was extracted from virus-free simian virus 40 (SV40)-transformed hamster, mouse, and monkey cells and was inoculated into simian cells in the presence of diethylaminoethyl (DEAE)-dextran; infectious SV40 was recovered by using DNA from cell lines which fail to yield virus by the fusion technique as well as from cell lines which readily yield virus by fusion. The rescued virus was identified as SV40 by three methods: (i) neutralization of plaque formation by specific antiserum; (ii) induction of synthesis of viral-specific antigens detected by immunofluorescence; and (iii) presence of papovavirus particles seen by the electron microscope. Treatment of the transformed cell DNA with deoxyribonuclease or omission of the DEAE-dextran prevented the rescue of virus. Large amounts of transformed cell DNA were required (>10 μg/culture of 10^6 cells) to effect rescue of SV40 by passage through monkey cells. A linear response was obtained between the input of DNA with inocula between 10 and 45 μg of DNA/culture and the yield of SV40 recovered. Biological activity was demonstrable irregularly when the transformed cell DNA was assayed directly in the presence of DEAE-dextran. The DNA induced plaque formation in about 50% of the trials as well as the synthesis of SV40 tumor and viral antigens in rare simian cells. The infectious DNA appeared to be associated with cellular DNA. The infectivity was found in the pellet of precipitated DNA obtained by the Hirt technique and was inactivated by boiling for 15 min. These properties are characteristic of linear cellular DNA and not of free, circular SV40 DNA.

Simian virus 40 (SV40)-transformed cells appear to be virus-free, although there is strong evidence that the transforming viral genome persists in an integrated state in the transformed cells (3, 5, 11, 40, 50, 52). Most studies detected multiple copies of the viral genome in each transformed cell, although the estimates varied as to the actual number of genome equivalents present. Recent work using deoxyribonucleic acid (DNA) reassociation kinetics (11) suggests that the number of SV40 genome copies per cell may be closer to one.

It is possible, under specified conditions, to recover small amounts of infectious virus from most SV40-transformed cell lines (3, 5). The most efficient method currently available for recovering SV40 involves the fusion of transformed cells with permissive cells mediated by inactivated Sendai virus (8, 12, 26, 49). However, this method succeeds in rescuing SV40 from only a rare cell (26, 49).

This report describes a new method for rescuing virus from SV40-transformed cells which involves the extraction of DNA from the transformed cells followed by passage through permissive simian cells. It presents additional evidence that the resident viral genome is associated with the cellular DNA. It is of interest that the "DNA-transfer" method also allows recovery of infectious SV40 from transformed cell lines which have previously failed to yield virus under any conditions.

MATERIALS AND METHODS

Cells. Primary green monkey kidney (GMK) cells were grown in Melnick's lactalbumin hydrolysate medium (M-H) supplemented with 2% fetal bovine serum (FBS), 0.075% sodium bicarbonate, 100 units of penicillin and 100 μg of streptomycin per ml (31). Two stable GMK cell lines were used: CV-1 (18), obtained from Saul Kit, and BSC-1 (16), kindly provided by Renato Dulbecco. The two cell lines had undergone about 135 and 120 passages, respectively, in our laboratory.

A summary of the properties of the transformed

cell lines used in this study is presented in Table 1. The TSV-5 cells (47), kindly supplied by S. S. Tevethia, are derived from a hamster tumor induced in vivo with SV40 and have been passed about 15 times in our laboratory. They contain SV40 tumor (T) antigen, no SV40 viral (V) antigen, and yield infectious SV40 following fusion with monkey cells (25). The $RIBS_{11}$ and SRHH cells are derived from kidney cells from Syrian-Romanian hybrid hamsters which were transformed in vitro by SV40. Both cell lines, kindly supplied by M. Nachtigal, contain SV40 T antigen and no SV40 V antigen; only the $RIBS_{11}$ cells were found to release virus following fusion with the CV-1 cells. The cells have been passed 25 to 40 times in our laboratory.

The H-50 cell line was derived from a hamster tumor induced in vivo by SV40 (1). It contains SV40 T antigen (35), tumor-specific transplantation antigen (TSTA) (20), viral DNA copies (27, 52), but no V antigen, and has been found to be virus-free in a number of different laboratories (30; 52; Butel, *unpublished observations*). The cells have been passed about 70 times in tissue culture.

The SV-3T3 cells (46) are an SV40-transformed mouse cell line obtained from Howard Green. The cells contain SV40 T antigen, no SV40 V antigen, and release SV40 by fusion. The transformed mouse kidney cell line, mKS-A, kindly supplied by S. Kit, was obtained by inoculating primary mouse (BALB/c) kidney cultures with SV40 (9). The cells contain SV40 T antigen, TSTA, and yield virus following fusion with CV-1 cells (24). Another transformed mouse cell line, VLM, kindly provided by S. S. Tevethia, was derived from BALB/c mouse embryo cells transformed in vitro by SV40 (Tevethia, *personal communication*). The cells contain SV40 T antigen and TSTA, but lack V antigen. The cell line has undergone approximately 15 passages in vitro.

The BSC-1-S cells, kindly supplied by Natan Goldblum, Jerusalem, are a clonal line derived from BSC-1 cells transformed by SV40 (28) and have been passed about 70 times in our laboratory. They contain SV40 surface (S) antigen and TSTA, but synthesize no V antigen under normal conditions. The cells release no infectious virus after fusion with susceptible cells (28, 37) and are resistant to superinfection by SV40 (4, 28, 37). The T-22 cell line (41) is derived from GMK cells transformed in vitro by irradiated T fraction (48) of SV40. These cells, kindly supplied by Hiroto Shimojo, Tokyo, and passed about 75 times in our laboratory, contain SV40 T antigen, no V antigen, and release no infectious virus. The T-22 cells are susceptible to superinfection by SV40 virions (4, 41).

Used as controls were cultures derived from tumors induced in hamsters by either dimethylbenzanthracene (Lausch, *unpublished observations*) or by a temperature-sensitive mutant of adenovirus type 31 (44). Both transformed cell lines, designated DMBA and Ad 31 ts (Tevethia and Butel, *unpublished observa-*

TABLE 1. *Properties of transformed cells employed in this study*

Cell line	Origin		Transforming agent	SV40-specific antigens[a]			Yield SV40 by fusion	Passages used in this study
	Species	Transformed		T	TSTA	V		
TSV-5	Hamster	In vivo	SV40	+[b]	ND	0	+	12–17
$RIBS_{11}$	Syrian-Romanian hybrid hamster	In vitro	SV40	+	ND	0	+	22–27
SRHH	Syrian-Romanian hybrid hamster	In vitro	SV40	+	ND	0	0	35–39
H-50	Hamster	In vivo	SV40	+	+	0	0	60–70
SV-3T3	Mouse	In vitro	SV40	+	ND	0	+	63–68
mKS-A	Mouse	In vitro	SV40	+	+	0	+	13
VLM	Mouse	In vitro	SV40	+	+	0	0	12–15
BSC-1-S	Monkey	In vitro	SV40	+	+	0	0	60–70
T-22	Monkey	In vitro	T-fraction of SV40 (irradiated)	+	ND	0	0	70–75
DMBA	Hamster	In vivo	DMBA	0	0	0	ND	13–15
Ad 31 ts	Hamster	In vivo	Adenovirus 31 ts	0	0	0	ND	13
SHL-7 (39)	Hamster	In vitro	PARA (39nT)-adeno 7	+	ND	0	ND	44

[a] T, Tumor; TSTA, tumor specific transplantation antigen; V, viral.
[b] +, Presence of antigen; 0, absence of antigen; ND, not done.

tions), respectively, lack SV40-specific antigens. The SHL-7 (39) cell line was established from adult hamster lung cells transformed in vitro by a "non-oncogenic" clone of a defective SV40-adenovirus hybrid, PARA (39nT)-adenovirus 7 (6). The cells possess SV40 T and S antigens, a hypodiploid stemline (32), and have been passed about 45 times in vitro.

All of the above stable and transformed cell lines were grown in Eagle medium (10) supplemented with 10% FBS, 10% Tryptose phosphate broth, 100 units of penicillin and 100 μg of streptomycin per ml, and 0.075% sodium bicarbonate.

Virus. SV40 was the Baylor reference strain; virus stocks were prepared as described previously (4).

Viral assays. SV40 was assayed by the plaque technique in BSC-1 cells growing in 60 by 15 mm plastic petri dishes. Tenfold dilutions of the virus were made in tris(hydroxymethyl)aminomethane (Tris) buffer, and 0.1-ml samples of the virus dilution were added to duplicate plates containing 0.3 ml of Tris buffer as carrier fluid. The virus was adsorbed for 2 hr at 37 C, and then 5 ml of overlay was added to each plate. The overlay consisted of Eagle medium supplemented with 10% FBS, 1% agar, 0.23% $NaHCO_3$, 100 units of penicillin per ml, 100 μg of streptomycin per ml, and 50 μg of mycostatin per ml. The plates were incubated at 37 C in an atmosphere of 5% CO_2. A second overlay containing a 1:20,000 dilution of neutral red in addition to the above supplements was applied 7 days later, and plaques were counted 12 to 16 days after the plates were inoculated.

Direct assays for plaque formation by DNA were carried out as described above, except that the DNA was mixed with an equal volume of a 1 mg/ml solution of diethylaminoethyl-dextran (DEAE-dextran) (34). Samples (0.2 ml) of the DNA-DEAE-dextran mixture were inoculated per plate and allowed to adsorb for 30 min at 37 C before the addition of the overlay.

Extraction of DNA: method 1. The Hirt (15) procedure with a phenol extraction step added was used. A 16-oz bottle of cells was washed twice with 0.01 M Tris (*p*H 7.4), and then 2.5 ml of 0.6% sodium dodecyl sulfate (SDS) in 0.01 M Tris and 0.001 M ethylenediaminetetraacetate (Tris-EDTA) was added and allowed to incubate for 20 to 30 min at 37 C. The lysate was poured into a thick-walled glass Sorvall tube and 5 M NaCl was added to a final concentration of 1 M. The tube was gently inverted about 10 times and stored overnight at 4 C. The mixture was centrifuged for 30 min at 12,000 rev/min at 4 C, and the supernatant fluid was removed. The pellet was resuspended with a capillary pipette in 1 ml of 1 × SSC (0.15 M NaCl, 0.015 M sodium citrate) and both the supernatant fluid and resuspended pellet were dialyzed for 24 hr against 1 × SSC at room temperature. The supernatant fluid was then stored at 4 C, and the resuspended pellet was treated as follows. One-tenth milliliter of a 1 mg/ml solution of Pronase (Calbiochem, Los Angeles, Calif.) (which had been self-digested by incubation at 37 C for 1 hr) was added to the mixture and incubated for 30 min at 37 C. The DNA was deproteinized by mixture with two volumes of redistilled phenol (which had been washed with Tris-EDTA), and the mixture was centrifuged at 10,000 rev/min for 30 min at 4 C. The top aqueous phase, containing the DNA from the Hirt pellet, was dialyzed against 1 × SSC for 24 hr at room temperature and stored at 4 C.

Method 2. Cell DNA was extracted by the Marmur (29) procedure. Cells in a 16-oz bottle were washed with Tris buffer, collected, and pelleted. A 2.5-ml amount of 0.5 M EDTA and 0.25 ml of 25% SDS were added, and the suspension was shaken until it was clear and viscous, at which time 5 M sodium perchlorate was added to a final concentration of 1 M. The suspension was deproteinized with two volumes of a 24:1 mixture of chloroform and isoamyl alcohol for 20 min and centrifuged at 5,000 rev/min for 10 min at 4 C and the aqueous layer was removed. The deproteinization steps were repeated five to seven times. The DNA was precipitated by slowly adding cold 90% ethanol, was spooled onto a glass rod, and was then dissolved in 1 × SSC. Ribonuclease (Worthington Biochemical Corp., Freehold, N.J.) was added to a final concentration of 50 μg/ml, and the mixture was incubated for 1 hr at 37 C. The deproteinization procedure was repeated three to five times to remove the ribonuclease. The aqueous layers were pooled, and the DNA was precipitated with cold ethanol, spooled onto a glass rod, and then dissolved in 0.1 × SSC for storage at 4 C.

Method 3. This method is a modification of that reported by Graham et al. (13). Cells were scraped from a 16-oz bottle after being washed with warm Tris buffer. The cells were resuspended in 10 ml of TNE (0.01 M Tris-hydrochloride, 0.1 M NaCl, and 0.01 M EDTA, *p*H 7.4), and 0.2 ml of DEOF (diethyl oxydiformate) and 0.1 ml of 0.5 M EDTA were added. The mixture was shaken 10 times; 10% sodium lauryl sarcosinate and 10% SDS were added to final concentrations of 0.5% and 0.2%, respectively. The mixture was incubated for 10 min at 37 C, at which time 0.05 g of TINS (triisopropyl naphthylene sulfonic acid, sodium salt) per ml was added and shaken well. An equal volume (about 12 ml) of PMQN (phenol–*m*-cresol mixture consisting of 500 g of phenol, 70 ml of *m*-cresol, 0.5 g of 8-hydroxyquinoline, and 55 ml of 0.15 M NaCl) was added and shaken gently in an ice bath. The mixture was centrifuged at 7,000 rev/min for 15 min at 4 C, the aqueous layer was removed, and deproteinization with PMQN was repeated eight to ten times until the yellow middle layer was no longer visible. The pooled aqueous layers were carefully layered with two volumes of cold 90% ethanol, and the precipitated DNA was then wound onto a glass rod and dissolved in 0.1 × SSC. Ribonuclease (500 μg) was added and the mixture was incubated for 30 min at 37 C; Pronase (50 μg) was then added and incubation was continued for 30 additional min. Deproteinization in an ice bath with PMQN was repeated once to remove the ribonuclease and Pronase. The aqueous layer was collected and treated with diethyl ether to remove the phenol, and a stream of nitrogen was bubbled into the mixture for 1 hr to

remove the ether. The DNA was then dialyzed against 0.1 × SSC for 48 hr and stored at 4 C.

Treatment with deoxyribonuclease: As a control, the DNA solutions were sometimes treated with 1 mg of DNase per ml (Nutritional Biochemical Corp., Cleveland, Ohio) at 37 C for 30 min.

Immunofluorescence techniques. The immunofluorescence techniques employed have been described previously (4, 36).

When transformed cellular DNA was assayed directly for ability to induce the synthesis of SV40 T and V antigens, GMK cells growing on 15-mm round cover glasses were exposed to 0.1 ml of a suspension of the test DNA mixed with an equal volume of a 0.2 mg/ml solution of DEAE-dextran, incubated at 37 C for 30 min, and then flooded with medium. Cultures were harvested 2 and 3 days later and stained as described above for T and V antigens, respectively.

Passage of transformed cell DNA in simian cells. A preparation of transformed cell DNA, containing approximately 250 μg/ml, was mixed with an equal volume of a 1 mg/ml solution of DEAE-dextran, and 0.2 to 0.4 ml of the DNA-DEAE-dextran suspension was then inoculated per culture containing about 10^6 simian (GMK or CV-1) cells. The inoculated cultures were incubated for 30 min at 37 C, the monolayers were washed gently with warm Tris buffer, culture medium was added, and incubation was continued at 37 C. About 12 days later, the cells were disrupted by alternate cycles of quick-freezing and thawing, cell debris was removed by low-speed centrifugation, and the supernatant fluid was tested for infectious SV40 by the plaque assay described above.

Fusion experiments. The fusion procedure described by Harris and Watkins (14) and modified by Dubbs and Kit (8) was used. A 1-ml amount of transformed cells (5×10^6 cells) was mixed with 1 ml of susceptible cells (1×10^7 cells), and the cells were pelleted by centrifugation. The cell pellet was resuspended in 1 ml of beta-propiolactone-inactivated Sendai virus (33) containing 4,000 hemagglutinating units, was held in ice for 10 min, and then shaken for 20 min at 37 C. The mixture was then washed with growth medium to remove the Sendai virus and resuspended in 5 ml of growth medium. Samples (1 ml) were distributed to each of five 60-mm Petri dishes, and 4 ml of fluid medium was added per dish. After overnight incubation at 37 C in a 5% CO_2 humidified incubator, the fluid medium was removed and the standard overlay used in the SV40 plaque assay was added. A second overlay containing a 1:20,000 dilution of neutral red was applied on the seventh day. Controls in each experiment included suspensions of each individual cell line exposed to Sendai virus as well as mixtures of the transformed and susceptible cells which were not treated with Sendai virus.

RESULTS

Attempts to rescue virus from SV40-transformed cells by the fusion technique. Following the procedure described above, attempts were made to recover virus from those SV40-transformed cells not previously tested by fusion with CV-1 cells using inactivated Sendai virus (Table 2). The $RIBS_{11}$ cells routinely released virus with an average yield from three experiments of 60 plaque-forming centers per 10^6 transformed cells. The SRHH cells failed to release any plaque-forming virus when treated under identical conditions. In confirmation of many previous experiments (30, 52; Butel, *unpublished observations*), the H-50 cells also appeared to be virus-free. The mKS-A cells, reported by Dubbs et al. (9) to release infectious SV40, were included as a positive control to ensure that the fusion system employed was adequate for recovering virus from transformed mouse cells as well as from transformed hamster cells. Under the same conditions, the VLM cell line failed to yield virus in three separate experiments. No plaques formed when the permissive CV-1 cells were treated with the inactivated Sendai virus preparation in the absence of transformed cells.

Recovery of virus by transfer of DNA from transformed cells to susceptible cells. DNA was extracted from a series of yielder and nonyielder transformed cell lines by three different extraction procedures described above. Large amounts (25 to 50 μg) of the DNA species, in the presence of DEAE-dextran, were then inoculated onto permissive GMK cells (10^6 cells/culture) as outlined in Materials and Methods. Plaque-forming virus was present 12 days later in lysates of GMK cultures exposed to DNA from the SV40-transformed cell lines (TSV-5, $RIBS_{11}$, SRHH, H-50, SV-3T3, VLM, and BSC-1-S). Typical yields of virus are summarized in Table 3. There were no appreciable differences in the amount of virus recovered using DNA prepared by the three different extraction procedures. The yields of plaque-forming virus were all low, ranging from 8.0×10^2 to 6.0×10^4 plaque-forming units (PFU)/10^6 cells.

The recovery of virus was abolished by pre-

TABLE 2. *Attempts to rescue virus from SV40-transformed cells by fusion with permissive cells in the presence of inactivated Sendai virus*

Cell line	No. of times virus rescued/no. of trials	Avg yield (PFC[a]/10^6 transformed cells)
$RIBS_{11}$	3/3	60
SRHH	0/3	0
H-50	0/1	0
mKS-A	1/1	12
VLM	0/3	0
CV-1	0/3	0

[a] PFC, Plaque-forming centers.

TABLE 3. *Recovery of virus from simian cells inoculated with DNA from SV40-transformed cells*

Source of cellular DNA	No. positive experiments/no. trials	Yield of virus (PFU[a]/10^6 cells)			
		First GMK cell passage of cellular DNA extracted by:			Second GMK cell passage (method 1)
		Method 1	Method 2	Method 3	
TSV-5	9/11	1.3×10^4[b]	4.5×10^3	3.0×10^3	2.6×10^6
$RIBS_{11}$	10/13	6.0×10^3	3.3×10^3	3.0×10^3	2.3×10^6
SRHH	12/15	4.6×10^4	5.5×10^3	6.0×10^4	3.7×10^6
H-50	5/10	9.0×10^2	2.4×10^3	1.1×10^3	1.0×10^4
SV-3T3	4/5	8.5×10^2	ND[c]	ND	2.0×10^4
VLM	6/10	1.0×10^3	1.1×10^3	1.1×10^3	6.0×10^4
BSC-1-S	2/4	8.0×10^2	1.1×10^3	ND	1.7×10^4
T-22	0/3	$<10^1$	$<10^1$	ND	$<10^1$
DMBA	0/4	$<10^1$	$<10^1$	$<10^1$	$<10^1$
Ad 31 ts	0/4	$<10^1$	$<10^1$	$<10^1$	$<10^1$
SHL-7 (39)	0/4	$<10^1$	$<10^1$	$<10^1$	$<10^1$
GMK	0/6	$<10^1$	$<10^1$	$<10^1$	$<10^1$
SV40-infected GMK (Hirt supernatant fluid)	6/6	4.1×10^6	3.5×10^7	3.0×10^6	ND

[a] PFU, Plaque-forming units.

[b] With each type of cellular DNA, no virus was recovered if the DNA were treated with deoxyribonuclease prior to passage or if the DEAE-dextran were omitted at the time the DNA was applied to the simian cells.

[c] ND, Not done.

treatment of the DNA with deoxyribonuclease, indicating that virus synthesis was initiated by the purified DNA. Since Pronase or ribonuclease treatment, or both, were used in the DNA extraction procedures (see Materials and Methods), those enzyme treatments were not inhibitory. In addition, omission of DEAE-dextran at the time of inoculation of the DNA into simian cells prevented the rescue of virus. No plaque-forming virus was recovered when DNA from the T-22, DMBA, Ad 31 ts, SHL-7 (39), or normal GMK cells was extracted and passed in parallel. These results suggest that the agent rescued by this technique was specifically recovered from SV40-transformed cells and was not introduced at some step in the extraction or passage procedures.

A secondary passage of the first GMK cell lysate through GMK cells increased the titer of the plaque-forming virus approximately 100-fold. Representative data are shown in Table 3. Samples which had appeared to be negative for infectious virus (those initially inoculated with deoxyribonuclease-treated DNA or DNA from non-SV40-transformed cells) remained negative after subsequent passage in GMK cells.

The data in Table 3 reveal that the DNA transfer method allowed the recovery of infectious virus from transformed cell lines which fail to yield virus by the fusion technique (SRHH, H-50, VLM and BSC-1-S) as well as from virus yielders (TSV-5, $RIBS_{11}$, and SV-3T3). In addition, virus was rescued from transformed cell lines of three different species of origin (hamster, mouse, and monkey).

The reproducibility of virus recovery by this technique is also indicated in Table 3. Some cell lines routinely yielded virus, such as SRHH did in 12 of 15 separate attempts. Virus was rescued from other cell lines, such as H-50 and BSC-1-S, in about 50% of the experimental trials.

Identification of the rescued virus as SV40. The plaque-forming virus rescued from the four SV40-transformed cell lines was identified as simian papovavirus SV40 (Table 4). The plaque-forming ability of the first GMK cell passage material was neutralized by treatment with specific SV40 antiserum prior to inoculation of the assay plates. These same samples were examined by electron microscopy and found to contain typical papovavirus-like particles.

Both SV40 T and V antigens were detected by immunofluorescence in GMK cells inoculated with either the first or second GMK cell passage material of DNA derived from the TSV-5, $RIBS_{11}$, SRHH, H-50, SV-3T3, VLM, and BSC-1-S cells. In addition, a number of plaques induced by the above passage material were picked, resuspended, and tested directly.

TABLE 4. *Identification of virus recovered from transformed cells as simian papovavirus SV40*

Source of cellular DNA[a]	First GMK cell passage			Plaques induced by first GMK cell passage: Induction of SV40 tumor and viral antigens
	Plaque formation neutralized by SV40 antiserum	Papovavirus particles by EM[b]	Induction of SV40 tumor and viral antigens	
TSV-5	+ (3/3)[c]	+	+ (3/4)	+ (3/4)
$RIBS_{11}$	+ (3/3)	+	+ (4/5)	+ (5/5)
SRHH	+ (3/3)	+	+ (5/5)	+ (4/4)
H-50	+ (1/1)	+	+ (5/5)	+ (4/4)
SV-3T3	+ (1/1)	+	+ (2/2)	+ (5/5)
VLM	+ (1/1)	+[d]	+ (4/4)	+ (3/4)
BSC-1-S	+ (1/1)	+	+ (3/3)	+ (2/3)
T-22	ND	0	0 (0/3)	ND
DMBA	ND	ND	0 (0/2)	ND
Ad31 ts	ND	ND	0 (0/1)	ND
SHL-7 (39)	ND	0	0 (0/2)	ND
SV40 virus stock	+	+	+ (6/6)	ND

[a] The data shown in this table was obtained by using DNA prepared by method 1 (a modification of the Hirt procedure).
[b] EM, Electron microscope; ND, not done.
[c] Numbers in parentheses: numerator, number of samples positive; denominator, number of samples tested.
[d] Results obtained with second GMK cell passage material.

The majority of the resuspended plaques also induced the synthesis of both SV40-specific antigens. The results summarized in Table 4 were obtained by using cell DNA which had been extracted by method 1. Similar results were obtained with lysates of cells infected with DNA extracted by methods 2 and 3. The absence of T and V antigen induction by material from cells inoculated with DNA from T-22, DMBA, Ad 31 ts, or SHL-7 (39) cells substantiates the conclusion that the virus was rescued from the SV40-transformed cells and was not introduced as a contaminant during the experiments.

Characterization of the "DNA transfer" rescue method. The phenomenon of the rescue of SV40 mediated by passage of transformed cell DNA through permissive simian cells was then characterized in more detail. The amount of transformed cell DNA required as inoculum to allow recovery of detectable quantities of infectious SV40 was determined by using DNA isolated from two transformed cell lines (SRHH and TSV-5) by method 1. The results are shown in Fig. 1. CV-1 cultures containing about 5×10^5 cells each were harvested 7 days postinoculation (pi) of the DNA. Large amounts of transformed cell DNA were required to rescue virus, as cultures inoculated with 10 μg (or less) of the DNA failed to synthesize infectious SV40. With inocula between 10 and 45 μg of DNA, proportionately larger amounts of SV40 were recovered 7 days pi. The addition of more than 45 μg of transformed cell DNA per culture did not result in increased yields of virus. In subsequent experiments, a minimum of 25 μg of transformed cell DNA per culture was employed as inoculum.

The optimal harvest time for the detection of virus rescued by the DNA transfer method was then determined. Replicate 1-oz bottle cultures of GMK cells were inoculated with 25 μg of transformed cell DNA (from the TSV-5, $RIBS_{11}$ and SRHH cell lines), and cell lysates were prepared and assayed for plaque-forming virus at 1, 7, and 14 days pi. A summary of the results is presented in Table 5. No virus could be detected 1 day pi of the DNA. Plaque-forming virus was present both 7 and 14 days pi, but the titers of SV40 were higher in the 14-day harvests, so this was selected as a standard harvest time for subsequent experiments.

Direct assays for biological activity of transformed cell DNA. Attempts were made to demonstrate biological activity for the transformed cell DNA species directly (without prior passage through GMK cells). A standard assay for infectious SV40 DNA, in which 25 to 50 μg of trans-

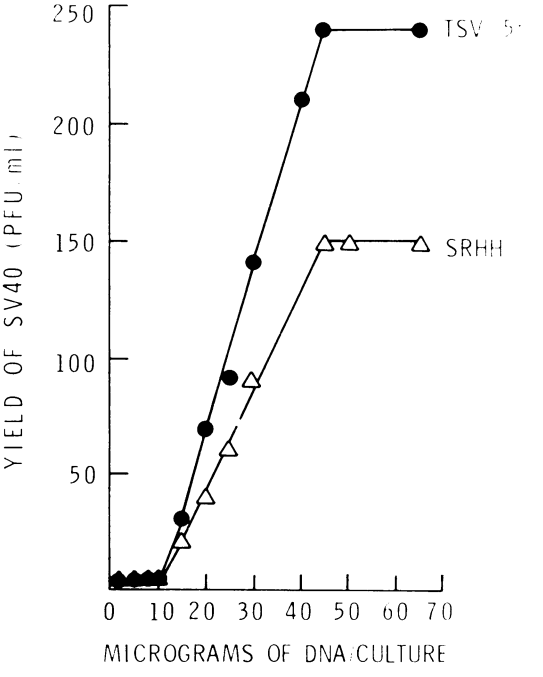

FIG. 1. *Relationship between amount of transformed cellular DNA inoculated per culture containing 5×10^5 CV-1 cells (in presence of DEAE-dextran) and yield of infectious SV40 recovered 7 days later. Both TSV-5 and SRHH are SV40-transformed hamster cell lines.*

TABLE 5. *Determination of optimal incubation period to detect virus rescue by the DNA transfer method*[a]

Source of cellular DNA[b]	PFU per 10^6 cells		
	1[c]	7	14
TSV-5	$<10^1$	2.0×10^2	3.0×10^4
$RIBS_{11}$	$<10^1$	5.0×10^1	3.0×10^4
SRHH	$<10^1$	2.0×10^2	2.0×10^4
GMK	$<10^1$	$<10^1$	$<10^1$
SV40-infected GMK (Hirt supernatant fluid)	$<10^1$	1.5×10^5	1.5×10^6

[a] A 25-μg amount of cell DNA inoculated per culture in this experiment.
[b] DNA species extracted by method 2.
[c] Days postinoculation.

formed cell DNA was inoculated per assay plate, was performed as described in Materials and Methods. Plaque formation was induced by the DNA preparations as illustrated by representative data in Table 6. The plaque-forming titers of the DNA from the TSV-5, $RIBS_{11}$, SRHH, H-50, SV-3T3, VLM, and BSC-1-S cells in the direct plaque assays ranged from 1.8×10^1 to 2.0×10^2 PFU/25 μg of DNA prepared by method 1. Similar values were obtained with DNA extracted by methods 2 and 3. No plaques were initiated by DNA extracted by the same procedures from the T-22, DMBA, Ad 31 ts, SHL-7 (39), and normal GMK cells. The latter DNA samples were the same as those which failed to release virus when passaged once through GMK cells (Table 3). The presence of DEAE-dextran was required; no plaques formed when equivalent amounts of transformed cellular DNA were inoculated in the absence of DEAE-dextran. With those DNA species which did induce plaque formation, positive results were obtained in about 50% of the trials.

Portions of the same DNA samples, in the presence of DEAE-dextran, were tested by immunofluorescence for the induction of SV40 T and V antigens. The results were variable (Table 6). Rare cells in the infected cultures were positive for SV40 T or V antigen in about half of the experiments attempted.

Evidence for association of infectious DNA with cellular DNA. Attempts were then made to ascertain the state of the infectious DNA in the transformed cell. It was important to establish whether any complete virus or free viral DNA was present. Three SV40-transformed hamster cell lines were employed for this analysis, and the results are summarized in Table 7. Cell lysates, prepared by disrupting 10^7 cells into 1 ml of medium, did not contain any virus, as evidenced by failure to induce plaque formation directly or after one passage in GMK cells.

When cells are extracted by the Hirt (15) procedure, the large molecules of cell DNA are precipitated and are found in the pellet, whereas molecules the size of free SV40 DNA remain in the supernatant fluid. All three transformed cell lines yielded infectious DNA in the Hirt pellet fraction, while the Hirt supernatant fluids from the same cells were found to be free of infectious viral DNA, yielding no virus directly or after one passage through GMK cells. The possibility that the infectious DNA was free viral DNA which was being trapped and mechanically carried into the precipitate with cellular DNA was discounted by using GMK cells infected 7 days earlier with SV40 virus. The Hirt pellet from this sample was negative for both plaque-forming ability and for the induction of synthesis of SV40 T and V antigens when assayed by immunofluorescence. All the infectivity appeared in the Hirt supernatant fraction obtained from the productively infected cells.

Linear cellular DNA is denatured when heated

TABLE 6. *Ability of DNA from SV40-transformed cells to induce plaque formation and the synthesis of SV40-specific antigens in simian cells in the presence of DEAE-dextran*

Source of cellular DNA	PFU/25 μg DNA[a]	No. positive experiments/ no. trials	Induction of SV40 T and V antigens[b]	No. positive samples/ no. samples tested
TSV-5	2.0×10^2	3/6	R+	4/6
$RIBS_{11}$	1.5×10^2	3/6	R+	3/6
SRHH	1.2×10^2	3/6	R+	3/6
H-50	1.8×10^1	2/5	R+	1/3
SV-3T3	5.0×10^1	2/3	R+	2/3
VLM	3.0×10^1	2/4	R+	1/3
BSC-1-S	1.8×10^1	1/2	0	0/2
T-22	0	0/2	0	0/2
DMBA	0	0/2	0	0/2
Ad 31 ts	0	0/2	0	0/2
SHL-7 (39)	0	0/2	0	0/2
GMK	0	0/6	0	0/6
SV40-infected GMK (Hirt supernatant fluid)	$>10^4$	6/6	+	6/6

[a] Data obtained using DNA extracted by method 1.
[b] R+, Presence of antigen in rare cell; +, presence of antigen; 0, absence of antigen.

TABLE 7. *Attempts to determine the state of the infectious DNA in the transformed cells*

Cell line	Sample tested	Sample should contain the following materials if present in the cells listed in column 1	Yield of SV40	
			Direct assay[a] (PFU/ml)	First GMK cell passage (PFU/10^6 cells)
TSV-5	Cell lysate	Complete virus	$<10^1$	$<10^1$
	Hirt pellet	Cellular DNA	2.0×10^2	2.0×10^3
	Hirt supernatant	Free SV40 DNA	$<10^1$	$<10^1$
	Marmur-extracted DNA[b]	Total DNA	8.0×10^1	4.5×10^3
	Marmur-extracted DNA, Δ 100 C, 15 min	Denatured linear DNA, intact circular SV40 DNA	$<10^1$	$<10^1$
$RIBS_{11}$	Cell lysate	Complete virus	$<10^1$	$<10^1$
	Hirt pellet	Cellular DNA	2.6×10^2	3.0×10^3
	Hirt supernatant	Free SV40 DNA	$<10^1$	$<10^1$
	Marmur-extracted DNA[b]	Total DNA	3.0×10^2	3.2×10^3
	Marmur-extracted DNA, Δ 100 C, 15 min	Denatured linear DNA, intact circular SV40 DNA	$<10^1$	$<10^1$
SRHH	Cell lysate	Complete virus	$<10^1$	$<10^1$
	Hirt pellet	Cellular DNA	2.0×10^2	2.3×10^3
	Hirt supernatant	Free SV40 DNA	$<10^1$	$<10^1$
	Marmur-extracted DNA[b]	Total DNA	4.0×10^2	2.0×10^3
	Marmur-extracted DNA, Δ 100 C, 15 min	Denatured linear DNA, intact circular SV40 DNA	$<10^1$	$<10^1$
SV40-infected GMK	Hirt pellet	Cellular DNA, trapped SV40 DNA	$<10^1$	$<10^1$
	Hirt supernatant	Free SV40 DNA	5.0×10^4	1.2×10^7
	Hirt supernatant mixed with Hirt pellet from control GMK cells, Δ 100 C, 15 min	Denatured linear DNA, intact circular SV40 DNA	5.0×10^3	1.2×10^7
Control GMK	Hirt pellet	Cellular DNA	$<10^1$	$<10^1$
	Hirt supernatant	Free SV40 DNA	$<10^1$	$<10^1$

[a] Assayed in presence of 1 mg of DEAE-dextran per ml.
[b] DNA extracted by method 2.

at 100 C for 15 min, whereas SV40 DNA retains its infectivity due to the superhelical circular configuration of the molecule (22). DNA species from the transformed cell lines were extracted by method 2 and samples were heated at 100 C in a boiling water bath for 15 min before inoculation of GMK cells. Heating abolished the infectivity of the transformed cellular DNA species, based on direct assays of the heated samples as well as assays of the first GMK cell harvests. Significantly, similar heating of the Hirt supernatant fraction from SV40-infected GMK cells mixed with a large excess of DNA from the Hirt pellet obtained from control GMK cells did not destroy the observed infectivity of that viral DNA. These data suggest that the infectious DNA which gives rise to mature SV40 virions is closely associated with the cellular DNA and does not exist in the transformed cell as free viral DNA analogous to that found in the complete virus particle.

DISCUSSION

There are several lines of evidence which suggest that the complete viral genome is present in all SV40-transformed cells, including the fact it has been possible occasionally to elicit the production of infectious SV40, by using a variety of techniques, from some of the transformed cell lines (5). Hamster tumors have occasionally contained virus in very low quantities, probably released spontaneously from rare cells in the tumor (39). Induction attempts with agents known to stimulate the release of lambda bacteriophage from

lysogenic *E. coli* sometimes released SV40 from transformed cells, but only from a rare cell and in very low titers (38). Physical contact between the transformed cells and permissive monkey cells was found to constitute a more efficient means of rescue of SV40 than the methods described above (12). The sensitivity of the cell contact method of virus rescue was increased by using inactivated Sendai virus to produce heterokaryons of the transformed and susceptible cells (8, 12, 26, 49). However, even with the fusion technique, some cell lines still fail to release any infectious virus, and those which do yield virus do so from only a rare cell.

It has been established that the transfer of DNA from lysogenic bacteria into nonlysogenic recipients results in the release of the resident bacteriophage genome, its subsequent replication, and the production of infectious progeny (42). Therefore, it was reasonable to postulate that a similar transfer of the transformed cell DNA into susceptible cells might be an effective means of recovering SV40.

The data presented in this paper show that, indeed, such transfer of transformed cell DNA to susceptible simian cells (in the presence of DEAE-dextran) does result in the recovery of infectious SV40. Large amounts of cellular DNA ($\backsim$25 μg) need to be introduced per culture containing about 10^6 simian cells in order to accomplish this rescue. These two requirements probably account for the negative results obtained by previous investigators attempting similar experiments. The success of this procedure is reminiscent of the results reported by Ito and Evans (17). They were able to induce papillomas in vivo with DNA extracted from papillomas induced by Shope papilloma virus in both cottontail and domestic rabbits. However, they gave no indication of the quantity of DNA in their preparations.

The DNA transfer method of virus rescue appears to be more efficient than cell fusion, as evidenced by the recovery of virus from the nonyielder cell lines. The fact that SV40 was recovered from the nonyielder transformed cells illustrates that the complete viral genome was present. It has frequently been suggested that those cell lines which fail to yield virus after fusion with simian cells were probably transformed by defective viruses, but the results reported in this communication cast doubt on that hypothesis. Of interest is the fact that the H-50 cell line has been carried in culture almost 10 years since the initial transforming event (1), and this is the first reported recovery of infectious SV40 from the cells. The recovery of virus from the BSC-1-S cells shows that it is possible for transformed permissive cells to carry a complete viral genome, although the possibility still exists that some cell lines may have been transformed by defective genomes. In fact, the T-22 cells may represent such a line as it was established after transformation by irradiated T fraction of SV40, and virus was not recovered from the cells in this study. Experiments of this type on other nonyielder cell lines, such as those described by Kit and colleagues (8, 21) or the abortively transformed clones of Smith et al. (43), would be informative.

The mechanism responsible for the recovery of virus after transfer of transformed cell DNA to susceptible cells is unclear at this time. Several possibilities may be considered. Firstly, it is possible that a specific excision of the integrated SV40 genome from the transformed cell DNA occurs under the direction of either a viral or host cell excision enzyme (or factors) in the simian cell, similar to the process which occurs with lambda bacteriophage in *E. coli* (42). Alternatively, random cleavage of the transformed cell DNA, either at the time of entry of the simian cell or some time thereafter, might occasionally allow the release of a complete SV40 genome which could then replicate. Random cleavage could be mediated by endogenous cellular nucleases or physical shearing, or both, which might occur during the process of entry of the DNA molecule. Thirdly, the transfer process might be analogous to "zygotic induction" (42) in that the integrated viral genome is removed from the presence of a specific repressor molecule. Evidence for an SV40-specific repressor has been presented by Cassingena and Tournier (7), but other investigators employing a variety of approaches and cell lines have been unable to substantiate the existence of such a repressor (2, 4, 19, 23, 37, 45).

The mechanism by which the DNA transfer method succeeds in rescuing virus from SV40-transformed cell lines which are nonyielders in fusion experiments is also obscure. Several different mechanisms can be postulated. (i) There may be certain integration sites in a tightly repressed region of the host chromosome from which it is not possible to rescue virus. This rigid host control, whatever its nature, may be broken down or removed when the cellular DNA is extracted and transferred. It is possible that a specific excision site is being exposed by these manipulations. (ii) A competent excision system may not be functional in the transformed cells, but may be present in the susceptible cells. Excision has previously been postulated to be the rate-limiting step in virus rescue (23). (iii) The physical manipulations in the technique may allow recombination to occur between defective viral genomes integrated at different sites in the transformed cell, resulting in the production of

infectious progeny virus. Although there are reports that multiple copies of the viral genome are contained in each transformed cell (11, 27, 52), the techniques used could not indicate whether or not some of the viral "equivalents" might be incomplete. The evidence is scant, but there is a suggestion that the multiple viral genomes are distributed among different chromosomes rather than being integrated in tandem (50, 51). Therefore, recombination between defective genomes is a possibility.

The fact that the integrated SV40 genome can be released and function efficiently in simian cells after introduction of transformed cell DNA raises the possibility of biological activity on the part of the overwhelming excess of hamster (or mouse or monkey) cell DNA. It seems possible that host cell markers may be transferred concurrently, in which case heterologous genes could be expressed and function in simian cells. The possibility that portions of the introduced donor DNA might become permanently associated with the chromosomes of the recipient simian cells is currently under investigation.

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