Rescue of Infectious Virus from Permissive Monkey Cells Containing Simian Virus 40 DNA Fragments

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Permissive TC7 cells were separately transfected with simian virus 40 (SV40) *EcoRI/Hap II A* (74% genome) DNA fragments and *EcoRI/Hap II B* (26% genome) DNA fragments in the presence of DEAE-dextran. Fusion of the progeny of recipient cells receiving the A fragment, TC7 (SV40/74) cells, with TC7 (SV40/26) cells, which had received the B fragment, resulted in SV40 rescue. TC7 (SV40/74 + 26) cells, which had simultaneously received both complementary subgenomes, either spontaneously produced SV40 upon subculture or yielded virus upon treatment with iododeoxyuridine. In addition, fusion of rat cells containing the *EcoRI/Hap II A* fragment with TC7 (SV40/26) cells resulted in SV40 rescue. Cytopathology, V-antigen production, neutralization, and electron microscopy were parameters used to verify that the rescued virus was SV40. No infectious virus was produced when the combinations of cells fused did not total a complete SV40 genome equivalent.

African green monkey kidney primary cells or continuous cell lines support the productive or lytic infection of simian virus 40 (SV40) or SV40 DNA (7, 26). Permissive indicator cells can detect virus replication in experiments designed to determine whether the virus or viral information is being maintained in transformed cells (13, 23, 41, 42). Transformed cells can be cocultivated with, or plated on, indicator cells to determine whether infectious virus is being produced (13). Fusion of transformed cells with permissive cells is commonly used to rescue virus from non-virus-producing transformants (13, 22, 23, 40, 41). The amount of virus produced by heterokaryons can be increased by preincubating the cells to be fused with the halogenated pyrimidine iododeoxyuridine (IUdR; 39). Halogenated pyrimidine treatment has also been used to induce production of virus or viral antigens by permissive cells that contain virus information (3, 14, 25, 34).

These studies were initiated to determine whether defined SV40 DNA subgenomes had any detectable biological activity in permissive cells and whether the subviral information could be maintained by the permissive cells in a potentially biologically active form. The restriction enzymes *EcoRI* and *Hap II*, from *Escherichia coli* and *Haemophilus aphrophilus*, respectively, were used to cleave SV40 DNA into an A fragment, constituting 74% of the genome and

including the entire early region, and a B fragment, constituting 26% of the genome and most of the late region. Each was separately transfected or both were cotransfected into the permissive African green monkey kidney cell line, TC7 (32). Subsequent rescue experiments and assays for virus-specific antigens showed that the subgenomes were maintained and could recombine to yield infectious virus.

MATERIALS AND METHODS

Sources of reagents and enzymes. Cell culture media, fetal calf serum, and anti-SV40 serum were obtained from GIBCO. Dimethyl sulfoxide, polyethylene glycol (PEG), and iododeoxyuridine (IUdR), were from Sigma Chemical Co. Restriction enzymes *EcoRI* and *Hap II* were from Miles Laboratories (Elkhart, Ind.), and *Hha*, *HindIII*, *Hpa I*, and *Bam I* were from Bethesda Research Laboratories (Bethesda, Md.).

Cells and virus. CV-1 cells (ATTC CCL 70) were from the American Type Culture Collection, Rockville, Md. The TC7 cell line (32) was a gift from M. Waite, University of Texas at Austin. Baby rat kidney (BRK) cells containing defined SV40 DNA fragments (1) were a gift from A. J. van der Eb and P. Abrahams, University of Leiden, The Netherlands. The H50 cell line (2) was a gift from J. Butel, Baylor College of Medicine, Houston, Tex.

Cells were grown at 37°C in Eagle minimum essential medium with Hanks salts supplemented with 10% fetal bovine serum (inactivated at 56°C for 30 min) and 292 µg of glutamine per ml. Antibiotics included in the growth media were either (i) penicillin (1,000 U/ml) and streptomycin (50 µg/ml) or (ii) gentamicin (20 µg/ml).

SV40 (strain PA-57) from the American Type Cul-

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ture Collection was used. Electrophoretic mobilities of fragments of component I SV40 PA-57 DNA from plaque-purified virus were identical to those of virion-purified DNA of SV40 strain 776 when the DNAs were cleaved with *EcoRI*, *Hap* II, *Bam* I, *Hha*, *Hind*III or *Hpa* I or when they were sequentially cleaved with *EcoRI* and *Hap* II or *Hind*III and *Hpa* I (data not shown).

Preparation and purification of SV40 DNA. CV-1 cells were grown in roller bottles and infected with SV40 at a multiplicity of infection of <1. SV40 DNA was prepared by the Hirt (18) procedure 74 h after infection. SV40 component I molecules were isolated from the Hirt supernatant essentially by the method of Sambrook et al. (33), except that the ethidium bromide concentration was 200 μ g/ml. Ethidium bromide was removed by three successive extractions with an equal volume of isopropanol (CsCl saturated) followed by dialysis against 10 mM Tris-hydrochloride-1 mM EDTA, pH 7.9. DNA concentrations were determined spectrophotometrically; absorbancy at 260 nm of 1.0 = 50 μ g/ml (11).

Generation and purification of endonuclease-derived fragments of SV40 DNA. Component I SV40 DNA (84 μ g) was treated sequentially with *EcoRI* and *Hap* II endonucleases (kindly supplied by R. J. Roberts), and the resulting fragments were separated by agarose slab gel electrophoresis (36). Two bands corresponding to the 74 and 26% genome fragments (10) were extracted from the gel, ethanol precipitated at -20°C , and subsequently dissolved in and dialyzed against SSC (SSC = 0.15 M NaCl + 0.015 sodium citrate). The entire procedure has been detailed in a preliminary communication (29).

Preparation and purification of DNA from uninfected TC7 cells. Hirt (18) DNA supernatant was obtained from one roller bottle culture of slightly subconfluent TC7 cells. The DNA was purified and centrifuged to equilibrium in CsCl-ethidium bromide gradients (33). A single band with an approximate buoyant density of 1.5547 g/cm³ was collected, extracted with isopropanol, and dialyzed against SSC.

Inoculation of cells with DNA. Fifty microliters of DEAE-dextran (1 mg/ml), followed by 50 μ l of DNA inoculum, was added to 50% confluent TC7 cells (ca. 2×10^4 to 3×10^4 cells) growing in 1-ml Leighton tubes containing 9- by 22-mm cover slips. The inoculated cultures were incubated for 60 min at 37°C ; the DNA/DEAE-dextran was removed and then washed gently with phosphate-buffered saline (PBS). Fresh growth medium was added, and incubation at 37°C was continued. Other TC7 cultures were inoculated with 50 μ l of 74% genome fragment SV40 DNA plus 50 μ l of 26% genome fragment SV40 DNA and 100 μ l of DEAE-dextran. Due to the relatively small yields of the SV40 DNA fragments, no direct determinations of DNA concentration were done. Since 60% recovery of the larger fragment and 80% recovery of the smaller fragment was expected (36), it was calculated that the cell cultures inoculated with the 74% genome fragment each received about 6.3 μ g and cultures inoculated with the 26% genome fragment each received 2.8 μ g. Hereafter, the TC7 cells that received the *EcoRI/Hap* II 74% SV40 subgenomic fragment, the 26% SV40 subgenomic fragment, and the 74% plus the 26%

subgenomic fragments of SV40 DNA will be referred to as TC7(SV40/74), TC7(SV40/26), and TC7-(SV40/74 + 26), respectively.

Control DNAs inoculated included SV40 components I and II and uninfected TC7 Hirt supernatant DNA. Mock-inoculated SSC and DEAE-dextran controls were also done.

Cell culture fluids and/or freeze-thaw lysates from all recipient cells were removed at 4, 7, and 8 days after inoculation and at frequent intervals thereafter. They were assayed on TC7 cells for SV40-induced cytopathology and production of SV40 V antigen as described below.

Assays for virus-specific antigens. (i) T antigen. Standard indirect immunofluorescence techniques (37) were used to detect SV40 T antigen. H50 (2) or 800Tu cells, derived by H. Hurtado from an SV40-induced transplantable tumor, were positive T-antigen controls.

(ii) V antigen. Hyperimmune rabbit anti-SV40 serum (GIBCO) was used in double agar immunodiffusion assays or as the primary antibody for indirect immunofluorescence assays.

Growth in soft agar. Upon reaching confluence, the DNA recipient cells were grown in agar suspension (27). Selected clones were individually harvested from the soft agar.

Treatment of cultures with IUDR. Various TC7(SV40/74), TC7(SV40/26), and TC7(SV40/74 + 26) cultures were grown for 24 h in complete culture medium containing 100 μ g of IUDR per ml or for 4 to 7 days in complete medium containing 30 μ g of IUDR per ml. Experimental and control cultures were maintained in complete medium and subcultured at 4- to 7-day intervals.

Cell fusion. The various combinations of cells fused are shown in Tables 2 through 6. Two techniques were used.

(i) Inactivated Sendai virus. Before fusion, the cells to be fused were subcultured and incubated for 24 h either in complete growth medium or in complete medium with IUDR (100 μ g/ml) to augment virus rescue (39-41). Cells were rinsed three times with PBS, trypsinized, counted, and then fused, using inactivated Sendai virus (2,000 hemagglutinating units/ml) according to standard techniques (39).

(ii) PEG. SV40 DNA fragment-containing monkey or rat cells (10^6 cells/fusion) were mixed with 10^6 TC7 cells in suspension. The cells were then centrifuged for 5 min at 1,000 rpm, resuspended in 1 ml of 50% PEG (wt/vol) in PBS, and incubated 30 min at 37°C . Volumes of complete medium equal to the volume of cell suspension were gradually added at 10-min intervals. After reaching a total volume of 16 ml, the fused-cell suspension was seeded into 75-cm² plastic flasks for virus rescue and put into four- or eight-chamber Lab-Tek slides for assay of T and V antigens.

(iii) Treatment of postfusion cultures. The percentage of cells fused was determined upon staining Leighton tube cultures with Giemsa and counting multinucleated cells. Postfusion cultures were maintained in growth medium with subculture every 4 days. Cell culture fluids from each subculture were stored at -90°C until analyzed for SV40 rescue.

Cocultivation. Various combinations of TC7-

(SV40/74), TC7(SV40/26), or TC7(SV40/74 + 26) cells were cocultivated, with or without TC7 cells. In each case, replicate cocultivations were done in which both types of cells either were not treated with IuDR or were incubated in medium containing IuDR (100 µg/ml) for 24 h before cocultivation. All cocultivated cells were subcultured every 5 to 7 days without IuDR at 37°C for 10 weeks. Cell culture fluids from each passage and selected freeze-thaw lysates were stored at -90°C until analyzed for SV40 virus rescue.

Determination of SV40 rescue. Cell culture fluids and/or freeze-thaw lysates were adsorbed onto TC7 cells for 1 to 3 h and then replaced with growth medium. The cultures were maintained for 20 to 30 days or until SV40 cytopathology and/or SV40 V antigen was observed.

Plaque assay and purification of rescued SV40. Serially diluted cell culture fluids from fusion cultures were adsorbed 1 to 2.5 h onto monolayers of TC7 cells in 60-mm petri dishes. Ten milliliters of 1% agar and 0.5% dimethyl sulfoxide (8) in growth medium was overlaid. A second overlay of 3 ml was added after 7 to 11 days, and another 3-ml overlay containing 0.02% neutral red was added after 13 to 19 days. Plaques were counted the following day, and viral concentration was expressed in PFU. Rescued SV40 was plaque purified by standard techniques.

Neutralization assay of rescued SV40 by hyperimmune rabbit anti-SV40 serum. Plaque reduction neutralization tests for SV40 were done, using hyperimmune rabbit anti-SV40 serum and standard neutralization techniques (16). Equal volumes of rescued virus (50 PFU/0.2 ml) and serially diluted rabbit anti-SV40 serum, normal rabbit serum, or PBS were incubated for 1 h at 37°C and then plaque assayed on TC7 cells.

Electron microscopy of plaque-purified rescued virus. Plaque-purified rescued virus and stock SV40 (strain PA-57) were pelleted by centrifugation at $69,500 \times g$ for 60 min at 4°C, resuspended in 0.2 ml of PBS, and stained for 1 min with a 2% solution of phosphotungstate, pH 7.0. The grids were examined on a Hitachi HS-8 electron microscope.

RESULTS

Effect of SV40 DNA and SV40 DNA fragments on permissive cells. All the recipient cells that were exposed to DEAE-dextran displayed a growth lag that eventually was overcome, and then the cells continued to grow. An initial growth lag followed by the appearance of focal growth areas, then subsequent overgrowth and pile-up of cells, was observed in the TC7(SV40/74), TC7(SV40/26), and TC7(SV40/74 + 26) cells. In the same time period, no overgrowth was observed in the mock-inoculated cells receiving DEAE-dextran alone or SSC alone. A slight morphological alteration and ultrastructural and nuclear abnormalities were observed upon subculture of the SV40 DNA fragment-inoculated cells, although

growth rates were not significantly different from that of parental cells (H. Hurtado, M. P. Moyer, and R. C. Moyer, in preparation).

Colony formation in soft agar. Clones of TC7(SV40/74), TC7(SV40/26), and TC7(SV40/74 + 26) were selected by colony formation in soft agar. Under these conditions, the DEAE-dextran mock-inoculated TC7 cells did not form colonies, whereas the H50 cell controls did. The soft-agar cloning efficiency, which averaged about 0.6%, did not vary appreciably among the TC7(SV40/74), TC7(SV40/26), and TC7(SV40/74 + 26) cells and was only slightly greater than that which can be obtained under certain conditions of rapid growth of TC7 cells alone (M. P. Moyer, unpublished observations; M. J. Tevethia, personal communication).

T antigen in DNA-inoculated cells. TC7 cells infected with component I or II SV40 DNA, H50 cells, TC7(SV40/74) cells, and TC7(SV40/74 + 26) cells exhibited typical nuclear T antigen, whereas TC7(SV40/26) or TC7 cells inoculated with cell DNA did not. Only a small percentage of the original recipient population of TC7(SV40/74) cells displayed T antigen. However, sublines of TC7(SV40/74) derived from soft-agar clones were from 30 to 90% T-antigen positive, depending upon the clone tested.

Tests for SV40 virions. The TC7 cells that received either the component I or the component II SV40 DNA inoculum were the only cells to immediately exhibit SV40 V antigen and cytopathology. The cytopathology was characterized by an increase in nuclear density, cytoplasmic vacuole formation, and rounding up and sloughing of the cells after 6 to 8 days. Neither V antigen nor cytopathology was evidenced in the TC7(SV40/74) or TC7(SV40/26) cultures or in TC7 cells inoculated with cell culture fluids from these cultures. Since SV40 component III molecules are also known to be infectious (38), no detectable single-enzyme cleavage products were in the original fragment preparations. The TC7(SV40/74 + 26) cells did not demonstrate SV40 cytopathology in the initial recipient culture, indicating that immediate complementation between co-infecting fragments did not occur. However, SV40 was rescued from most of the TC7(SV40/74 + 26) clones upon subculture and/or by treatment with IuDR (Table 1).

In a series of representative TC7(SV40/74 + 26) cultures (Table 1), virus was rescued from three of seven lines (clones 11, 14, and 18) upon subculture without IuDR induction and from four of five lines with IuDR induction (uncloned 530 and clones 13, 14, and 16). No infectious

virions were produced in up to eight subcultures of the selected cloned TC7(SV40/74) or TC7(SV40/26) cells after treatment with IUdR.

Efficiency of fusion. Cultures Giemsa stained 24 h after they were fused by using inactivated Sendai virus revealed 50 to 60% multinucleated cells. PEG-mediated fusion efficiency was 80% or greater.

Rescue of SV40 from heterokaryons. Infectious virions were successfully rescued by fusion of TC7(SV40/74) cells with TC7(SV40/26) cells (Table 3), fusion of BRK(SV40/74) with

TC7(SV40/26) cells (Table 2), or fusion of TC7(SV40/74 + 26) with TC7 cells (data not shown).

Rescue was evidenced by cytopathology and V-antigen production in the heterokaryon cultures and/or in TC7 cells infected by cell culture fluids and/or freeze-thaw lysates from the heterokaryon cultures (Tables 2-6). In addition, in the cases of fusions 1-005 and 1-012, the rescued infectious virus was plaque purified twice on TC7 cells and was verified as SV40 by V-antigen and virus neutralization.

TABLE 1. *Rescue of SV40 from TC7(SV40/74 + 26) cells*

Clone no.	Passage no.	T Antigen ^a	CPE ^b in culture	V Antigen	IUdR induction	Rescue
Uncloned 530	6, 7, 10	± (pN)	—	—	NA ^c	—
	10 (IUdR)	+3 (N)	+	+	+	+
11	6	+	+	+	ND ^d	+
12	6	—	—	—	—	—
	6 (IUdR)	—	—	—	—	—
13	5, 9, 12	+1 (N)	±	—	NA	—
	5 (IUdR)	+1 (N)	+	+	+	+
16	9, 12	+3 (N)	—	—	NA	—
	12 (IUdR)	+3 (N)	+	+	+	+
14	6	+2 (N; pN)	+	+	NA	+
	8	+2 (N)	+	—	NA	—
	12	+3 (N)	—	—	NA	—
	12 (IUdR)	+4 (N)	+	+	+	+
18	4	+3 (N)	+	+	ND	+

^a Antigen detected with anti-T sera. pN, Perinuclear; N, nuclear.

^b Cytopathic effect.

^c NA, Not applicable.

^d ND, Not done.

TABLE 2. *Rescue of SV40 by fusion of BRK(SV40/74) and TC7(SV40/26) cells*

TC7(SV40/26) clone no.	Fusion no. ^a	Total no. of passages ^b	Cytopathic effect		V antigen		SV40 rescue
			Heterokaryon passage ^c	Inoculated TC7 passage ^d	Heterokaryon passage ^e	Inoculated TC7 passage ^f	
6	3-113	7	± (1) ^g	+	— (1)	+	+
6, TC7 ^h	4-125	2	± (1)	+	+	+	+
9	3-106	12+	+	+	+	+	+
	3-107	8	+	+	+	+	+
	3-108	5	+	+	+	+	+
UcMISF ⁱ	3-101	10	+	+	+	+	+

^a Five fusion series were done. These are designated by the numbers preceding the dash. The numbers following the dash are the actual fusion experiment numbers. In fusion series 1 and 2 Sendai virus was used to mediate cell fusion. In series 3, 4, and 5 polyethylene glycol was used.

^b Total number of passages of the heterokaryon culture before the experiment was terminated.

^c Earliest heterokaryon passage number at which the culture displayed obvious cytopathology.

^d Earliest passage number of cell culture fluids from the heterokaryon culture at which cytopathology could be induced in permissive TC7 cells.

^e Earliest passage at which V antigen was detected in the culture.

^f Earliest passage number of cell culture fluids from the heterokaryon culture at which V antigen could be detected in the inoculated TC7 cells.

^g Passage number in parentheses.

^h Fusion of BRK(SV40/74) and TC7(SV40/26) C-6 and TC7 cells.

ⁱ An uncloned TC7(SV40/26) culture. Footnotes a-g and i also apply to Tables 3-6.

TABLE 3. *Rescue of SV40 by fusion of TC7(SV40/74) and TC7(SV40/26) cells*

Cells fused		Fusion no.	Total no. of pas- sages	Cytopathology		V antigen		SV40 res- cue	
TC7(SV40/ 26) clone no.	TC7(SV40/ 74) clone no.			Heterokar- yon passage	Inoculated TC7 passage	Heterokar- yon passage	Inoculated TC7 passage		
6	18	1-001	11	+ (10) ^a	+ (6)	ND ^a	+ (10)	+	
		2-069	5	+ (4)	+ (4)	ND	+ (4)	+	
		4-138	6	± (2)	+ (2)	+ (2)	+ (1)	+	
	44	1-024	10	+ (9)	± (4)	+ (8)	+ (4)	+	
		2-066	5	+ (5)	+ (4)	+ (5)	+ (4)	+	
		4-143	6	± (2)	+ (2)	+ (2)	+ (1)	+	
		2-063	11	+ (10)	+ (4)	+ (10)	+ (4)	+	
	68	2-068	12	+ (11)	+ (2, 5)	+ (12)	+ (2)	+	
	9	44	1-035	10	+ (9)	± (4)	+ (6)	+ (4)	+
		2-065	12	+ (11)	+ (4, 11)	+ (12)	+ (4)	+	
	68	1-021	11	+ (9, 10)	± (9)	+ (6)	ND	+	
		35	44	1-005	5	+ (4)	+ (1)	ND	+ (1)
			2-034	8	± (1)	± (4)	+ (6)	+ (4)	+
46	44	1-007	5	+ (4)	+ (1)	ND	+ (1)	+	
52	18	2-070	11	+ (7, 11)	+ (4)	+ (8)	+ (4)	+	
		44	1-002	5	+ (4)	+ (2)	ND	+ (2)	+
		2-067	12	+ (11)	+ (4)	+ (4)	+ (4, 5)	+	
		68	1-022	11	+ (10)	+ (4)	+ (6)	+ (4)	+
		18	1-020	10	+ (9)	+ (4)	+ (4)	+ (4)	+
	UcMISF	44	1-023	9	+ (8)	± (4)	ND	+ (4)	+
		68	1-036	9	+ (8)	± (4)	+ (4)	+ (4)	+

^a ND, Not done.TABLE 4. *Inability to rescue SV40 by fusion of TC7(SV40/74) or TC7(SV40/26) cells with TC7 cells only*

Clone no.	Fusion no.	Total no. of passages	Cytopathology		V antigen		SV40 rescue
			Heterokar- yon passage	Inoculated TC7 passage	Heterokar- yon passage	Inoculated TC7 passage	
TC7(SV40/74)							
18	2-079	10	— (9)	— (4)	— (10)	— (4)	—
44	1-037	11	± (0), — (2)	± (4)	— (4)	— (4)	—
	2-078	8	± (8)	— (4)	— (6)	— (4)	—
68	4-144	6	± (2), — (5)	— (5)	— (4)	— (4)	—
	1-029	8	— (8)	± (4)	— (4)	— (4)	—
	2-077	10	— (10)	— (4, 9)	— (3)	— (11)	—
	3-117	6	± (2), — (5)	— (5)	— (4)	— (2)	—
	4-139	6	± (2), — (5)	— (5)	— (4)	— (4)	—
TC7(SV40/26)							
6	2-080	9	— (9)	— (4, 9)	— (6)	— (9)	—
9	1-039	9	— (8)	— (8)	— (4)	— (8)	—
	2-081	10	— (9)	— (9)	— (5)	— (9)	—
52	1-028	10	— (9)	— (9)	— (4)	— (9)	—
	2-082	7	— (6)	— (4)	— (6)	— (4)	—
56	5-150	6	— (5)	— (4)	— (4)	— (4)	—

There was considerable variability in the number of possible subcultures of postfusion heterokaryons before cytopathology prevented further subculture (Tables 2 and 3). This averaged four to seven subcultures (16 to 28 days), with a minimum of one subculture and a maximum of eight subcultures. Generally, virus production by the heterokaryon cultures was detected earlier by inoculation of TC7 cells and

assay for V antigen than by assay of V-antigen production in the heterokaryon culture itself.

No infectious virions were rescued after 6 to 12 subculture passages following fusion of cells that should not contain a complete SV40 DNA equivalent (Tables 4-6). The lack of virus rescue was assessed by no observable cytopathology or V-antigen production either in the postfusion heterokaryon cultures or in the TC7 cells inoc-

TABLE 5. *Inability to rescue SV40 by fusion of different clones of TC7(SV40/74) with each other or TC7(SV40/26) with each other*

Clones fused	Fusion no.	Total no. of passages	Cytopathology		V antigen		SV40 res- cue
			Heterokar- yon pas- sage	Inoculated TC7 pas- sage	Heterokar- yon pas- sage	Inoculated TC7 pas- sage	
TC7(SV40/74)							
18 + 44	1-031	8	— (8)	— (4)	± (4)	— (4)	—
	2-071	10	— (9)	— (6, 10)	— (9)	— (9)	—
18 + 68	1-030	8	— (8)	— (4)	± (4)	— (4)	—
	2-073	8	± (7)	— (4)	— (7)	— (7)	—
44 + 68	1-027	7	— (7)	— (4)	± (4)	— (4)	—
	2-072	10	— (9)	— (4)	± (4)	— (4)	—
TC7(SV40/26)							
6 + 9	2-074	9	— (8)	— (6)	— (8)	— (8)	—
	2-076	10	— (9)	ND ^a	— (4)	— (9)	—
9 + 52	1-026	9	— (8)	— (7)	— (8)	— (8)	—
	2-075	10	— (9)	— (10)	— (10)	— (9)	—
52 + UcMISF	1-033	9	— (8)	— (4, 7)	± (4)	— (4)	—

^a ND, Not done.TABLE 6. *Inability to rescue SV40 by fusion of SV40 DNA fragment-containing monkey and rat cells whose viral genomes do not total a complete genome equivalent*

Rat cells	Monkey cells	Fusion no.	Total no. of passages	Cytopathology		V antigen		SV40 rescue
				Heterokaryon passage	Inoculated TC7 passage	Heterokaryon passage	Inoculated TC7 passage	
BRK(SV40/74)	TC7	4-124	3	± (1), — (2)	— (2)	— (2)	± (2)	—
		3-116	6	± (1)	— (5)	— (1), — (5)	— (5)	—
	TC7(SV40/74)							
	Clone 44	3-105	12	± (2, 12)	— (11)	— (12)	— (12)	—
	Clone 68	3-114	10	± (1)	— (9)	— (1)	— (2)	—
BRK(SV40/59)	TC7	3-115	8	± (1)	— (6)	— (1)	— (2)	—
		3-111	4	± (1), — (4)	— (3)	— (4)	— (3)	—
	TC7(SV40/74)	4-122	3	± (1), — (3)	— (2)	— (2)	± (2)	—
		3-104	15	± (1), — (2)	— (10)	— (10)	— (10)	—
	TC7(SV40/26)	3-112	2	+ (1)	ND ^a	— (1)	ND	—
		4-121	3	± (1)	— (2)	— (2)	— (2)	—
	UcMISF	3-103	10	± (1), — (2)	— (10)	— (10)	— (10)	—

^a ND, Not done.

ulated with supernatant fluids or terminal freeze-thaw lysates from the heterokaryon cultures.

Contrary to the results reported by others (6, 42) we observed the rescue of infectious virus after the fusion of H50 cells and TC7 cells (M. P. Moyer and R. C. Moyer, in preparation). Four to five subcultures (16 to 20 days) of the post-fusion heterokaryons were required, however, before rescue of infectious virus was demonstrable.

A cytopathic effect resembling that caused by SV40 was observed in some heterokaryon cul-

tures derived from fusions between cells containing the 74% genome fragment and permissive TC7 cells. This cytopathic effect was not lethal to all of the cells, and the cultures were capable of continued passage. In addition, SV40 V antigen was not demonstrable in these heterokaryon cultures. Infectious SV40 was not responsible for the observed cytopathology, because supernatant fluids and/or freeze-thaw lysates from these cultures were inoculated onto TC7 cells without resulting in cytopathology or SV40 V-antigen production. Abrahams et al. (1) also observed a cytopathic effect after fusion of SV40 74% ge-

nome-transformed BRK cells and permissive BSC-1 cells.

Cocultivation. Five separate cocultivations of TC7(SV40/74) and TC7(SV40/26) cells failed to produce infectious virus (data not shown) after 10 weeks of growth (17 subcultures). No cytopathology was observed for any of the five cocultivation studies when cell culture fluids or freeze-thaw lysates were inoculated onto TC7 cells. This observation remained true regardless of whether the cocultivated cells were treated with IUdR. Cocultivation of TC7 with cloned and uncloned TC7(SV40/74 + 26) cell lines resulted in virus rescue from cultures that were spontaneously producing virus (clones 11, 14, and 18; see Table 1), but not those in which the virus was maintained in a latent, albeit inducible by IUdR, form (clones 13, 14, and 16 and uncloned 530).

Neutralization of rescued SV40 by hyperimmune rabbit anti-SV40 serum. Virus rescued from two separate fusions (1-005 and 1-012) after plaque purification were tested for reactivity to anti-SV40 serum. The expected number of plaques (50/plate) was observed in assays of rescued virus alone. In assays of mixtures of rescued virus and normal rabbit serum, there was a slight reduction (<10%) in plaque number. No plaques were observed in assays from solutions of rescued virus and hyperimmune rabbit anti-SV40 serum up to a 1:1,000 dilution of antiserum.

Electron microscopy of plaque-purified rescued virus. When virus rescued from fusions 1-012 and 1-005 was compared with stock SV40 by electron microscopy, the rescued, plaque-purified virus resembled the stock virus in having typical papovavirus size and morphology (data not shown).

DISCUSSION

More than 20,000 integrated SV40 genome equivalents occur during productive infection (19), although this possibly is a high estimate due to trapping of nonintegrated SV40 DNA oligomers in the high-molecular-weight fraction (28). Kuhn et al. (24) have evidence that the integrated SV40 DNA sequences in infected CV-1 cells consist of subgenomic fragments and that these fragments are either directly adjacent to, or only separated by a short segment from, reiterated host DNA sequences. These studies support the concept that permissive cells readily integrate subgenomic fragments of SV40 DNA at a larger number of sites than do semipermissive or nonpermissive cells. Several studies indicate that nonpermissive and semipermissive

cells will stably accept only a few proviral genomes (9, 12, 17, 31), which may be integrated as complete or fragmented genomes (4, 5, 21, 33).

Perhaps only permissive cells possess integration sites (or a greater number of sites) capable of accepting late virus genes when they are not accompanied by early genes. This interpretation is supported by the studies comparing SV40 DNA fragment equivalents per diploid quantity of transformed mouse cell DNA (21). The *EcoRI/Hap I* (27%) B fragment, which is essentially identical to the *EcoRI/Hap II* (26%) B fragment, was represented two to six times less than other *EcoRI/Hap I* fragments in the remainder of the viral genome. Only certain specific fragments, derived from the early region of the tumor virus genome, could induce the transformed state in nonpermissive rat cells (1, 15). However, no studies have been done to determine whether the fragments that were not active inducers of transformation were maintained in the recipient cultures (A. J. van der Eb, personal communication).

Whether the TC7 cells, which received the SV40 subgenomes in these studies, are transformed is a debatable issue (29), based on an arbitrary definition of what constitutes a transformed cell. The TC7(SV40/74) and TC7(SV40/26) cells, in contrast to the DEAE-dextran mock-inoculated cultures, were capable of overgrowth under crowded conditions and grew in soft agar—both recognized properties of transformed cells (27, 35). Comparison of the properties typical of transformed cells between the TC7(SV40/74) and TC7(SV40/26) cell lines and the normal TC7 parent cell line is hampered by the fact that the TC7 cells elicit several properties of transformed cells, most notably, infinite growth transformation (30). Under certain conditions, TC7 cells and TC7 mock-inoculated cells can overgrow the monolayer and grow in soft agar (M. P. Moyer, unpublished observation; M. J. Tevethia, personal communication). TC7(SV40/74) and TC7(SV40/74 + 26) cells expressed typical nuclear T antigen, whereas the TC7(SV40/26) cells did not. In addition, the TC7(SV40/74) cells were weakly tumorigenic when injected into newborn hamsters (Hurtado et al., unpublished observations). Examination of other characteristics of various TC7(SV40/74) and TC7(SV40/26) clones (e.g., nuclear morphology, ultrastructure, and superinfection resistance; Hurtado et al., in preparation) indicate that a permanent, heritable change has occurred. However, it cannot be ruled out that subpopulations of TC7 cells that have some characteristics of transformed cells were selected

with the aid of DEAE-dextran toxicity. Further studies are in progress to clarify and expand these observations.

Since cocultivation of TC7(SV40/74) and TC7(SV40/26) cells did not result in virus rescue, the subviral determinants were not readily transferred from cell to cell in monolayer culture, as has been observed in other systems (13). This contrasted to rescue by cocultivation of some TC7(SV40/74 + 26) cells with indicator TC7 cells. The additional studies of the TC7(SV40/74 + 26) cells showed that a complete virus genome could be present with or without concomitant virus production by the permissive cells.

The various clones of TC7(SV40/74) and TC7(SV40/26) that yielded SV40 upon fusion had undergone from 5 to 11 subcultures from the initial transfection event, including the cloning procedure to the fusion experiments. That subculture is a prerequisite for rescue from some clones suggests that multiple rounds of DNA synthesis may be needed for the rare recombination event to occur.

We have further verified (unpublished observations) the maintenance of the viral subgenomes by using the nick-translation blot hybridization technique with class I, II, and III restriction enzymes (5).

The viral information is maintained in the high-molecular-weight Hirt (18) pellet fraction, since no viral sequences are detected in the Hirt supernatant DNA concentrated from 10^6 to 10^8 TC7(SV40/74) or TC7(SV40/26) cells (C. Ng, M. P. Moyer, and R. C. Moyer, unpublished observations).

The fact that infectious virions can be rescued by the fusion of two cell lines, each of which, many generations previously, had been transfected by a complementary subgenomic fragment of SV40 DNA, demonstrates some interesting capabilities of defective viral DNA introduced into a permissive cell: (i) the defective viral DNA can enter intact (or reversibly dispersed) into a stable association with the recipient cell; (ii) the defective viral DNA can be transmitted from the recipient cell to daughter cells; and (iii) the defective viral DNA remains potentially biologically active in the daughter cells and can continue to express some of the genetic information it contains (such as T antigen) as well as alter some of the growth properties of the host cell.

The potential of permissive cells to become productively infected whenever a resident defective viral genome is rescued presents an interesting conceptual model for latent, persistent, and recurrent viral diseases.

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