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Properties of Permissive Monkey Cells Transformed by UV-Irradiated Simian Virus 40

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African green monkey cells (CV1 line) were infected with UV-irradiated simian virus 40 (SV40), and permissive lines of stably transformed cells were established. These cell lines display the SV40 T-antigen and the growth characteristics typical of nonpermissive transformed cells (e.g., reduced cell density inhibition, reduced serum dependence, ability to overgrow normal cells, and colony formation in soft agar). The level of permissiveness to superinfecting SV40 is fully comparable with that of nontransformed CV1 and BSC-1 lines. The transformed monkey lines also support SV40 plaque production under agar. By C_0t analysis, the transformed permissive cells contain, on an average, 1 to 2 SV40 genome equivalents, and the majority of the viral sequences are associated with the high-molecular-weight cellular DNA. No spontaneous production of infectious SV40 has been observed. The transformed permissive monkey cells failed to support the replication of SV40 *tsA* mutants at the restrictive temperature. To account for this, it is suggested that the gene *A* product has separate functions for transformation and initiation of viral DNA synthesis, and only the former function is expressed in the transformed permissive monkey cells.

Transformation of permissive monkey cells by simian virus 40 (SV40) often results in loss of permissivity (reviewed in reference 27). This probably occurs because the putative transformed cells are continually exposed to SV40 such that permissive transformed cells are killed and only nonpermissive transformants survive. When precautions have been taken to limit reinfection and consequently to reduce the selection pressure for nonpermissive cells, transformed monkey cells with varying degrees of permissiveness have been obtained (27, 29, 30). We were interested in establishing new lines of SV40-transformed monkey cells, which fully retain their sensitivity to superinfection, for the following reasons. It may be possible to catalogue and analyze the functions expressed by integrated viral genomes by measuring the ability of different transformed cell populations to support the replication of defined deletion and temperature-sensitive (*ts*) viral mutants. Furthermore, such transformed cells would be useful for cloning defective virus variants. In addition, transformed permissive cells would provide a convenient system for studying recombination events between exogenous (superinfecting) viral genomes and endogenous chromosomally integrated viral genes.

In the present study we describe the development and properties of three lines of monkey CV1 cells, transformed by UV-irradiated SV40, which have retained a high sensitivity to superinfection. We also report that none of the three permissive transformed monkey lines supports the replication of SV40 *tsA* mutants at the restrictive temperature. To explain this lack of detectable complementation by the resident SV40 genome, we suggest that the cells were transformed by virus that acquired a mutation affecting the function for viral DNA synthesis but not the function for transformation. In subsequent publications (Y. Gluzman, E. L. Kuff, and E. Winocour, manuscript in preparation; T. Vogel, Y. Gluzman, and E. Winocour, manuscript in preparation) we will present evidence for recombination between the endogenous viral genes of the transformed permissive monkey cells and a superinfecting SV40 *tsD* mutant.

MATERIALS AND METHODS

Cells and viruses. Cells of the BSC-1 and CV1 monkey lines were grown in reinforced Eagle medium with 10% calf serum. The standard wild-type SV40 used was a plaque-purified stock of strain 777 grown from a limiting dilution (18). The SV40 *tsA209* and *tsA239* mutants (7) were obtained from R. G. Martin; *tsA30* (31) was obtained from P. Tegtmeyer. Virus was titered by plaque assay, as previously described (18), except that the nutrient agar overlay contained 0.4 μg of dexamethasone per ml

(22). Although it is not essential, the addition of dexamethasone helps to maintain the integrity of both the normal and transformed CV1 cell sheets under the agar overlay.

Transformation of monkey CV1 cells. Subconfluent cultures of CV1 or BSC-1 cells, containing 10^6 cells per 6-cm-diameter plate, were infected with 1 ml of a 1:5 dilution of a UV-irradiated, plaque-purified SV40 stock (strain 777). Two milliliters of virus, in a 6-cm-diameter plastic plate, was irradiated for 15 min at a distance of 22 cm from a Philips TUV 15-W germicidal lamp. The titer of the stock was 10^9 PFU/ml prior to irradiation and 10^5 PFU/ml after irradiation. At 18 to 20 h after infection, the cells were trypsinized, diluted, and plated in soft agar (19); 2.5 ml of 0.33% nutrient agar mix containing 150,000 infected cells and 1 to 2% anti-SV40 rabbit serum (Grand Island Biological Co., Grand Island, N.Y.) was plated on top of a 7-ml basal layer containing 0.5% nutrient agar mix. Every 10 days, 2.5 ml of fresh 0.33% agar mix containing 1% anti-SV40 serum was added. Readily visible colonies of cells appeared in the soft agar overlay after a period of 1 to 1.5 months. These were isolated with a Pasteur pipette and grown up to mass culture for further investigation.

Immunofluorescence tests. The SV40-infected or -transformed cells, grown on cover slips, were fixed with acetone-methanol (1:1) for 1.5 min at room temperature and then directly stained with fluorescein-conjugated antisera. Anti-SV40 T-antigen serum, from hamsters bearing virus-free SV40-induced tumors, was purchased from Flow Laboratories, Irvine, Scotland.

To prepare anti-SV40 V-antigen serum, rabbits were inoculated with a preparation of SV40 capsids ("empty shells") purified by equilibrium centrifugation in cesium chloride density gradients (33) and UV irradiated for 20 min as described above. The inoculation schedule, both with and without Freund adjuvants, and the conjugation with fluorescein isothiocyanate are detailed elsewhere (10, 11). The SV40 anti-V-antigen serum showed no reactivity against the SV40 T-antigen.

Virus DNA synthesis. Virus DNA synthesis was measured by the incorporation of [^{3}H]thymidine (TdR) into acid-precipitable (5% trichloroacetic acid) material in the Hirt supernatant fraction (15). Control experiments showed that [^{3}H]TdR incorporation into acid-precipitable material in the Hirt supernatant fraction correlates well with the incorporation of [^{3}H]TdR into supercoiled SV40 DNA isolated from the same fraction by equilibrium centrifugation in cesium chloride-ethidium bromide gradients and band sedimentation in alkaline cesium chloride gradients (17). To correct for variations in the number of cells per culture, the incorporation data were (where indicated) normalized to the amount of OD_{260} (optical density at 260 nm) material in the Hirt pellet fraction, dissolved by boiling for 15 min in water.

Fractionation of cellular DNA. Cellular DNA was extracted and fractionated into high- and low-molecular-weight DNA by the Hirt lysis procedure (15), except that the high-molecular-weight fraction ("Hirt pellet") was separated from the low-molecular-weight fraction ("Hirt supernatant") by centrifugation at 30,000 rpm for 1 h at 4°C in a Spinco 35 rotor. The Hirt supernatant fraction was treated with predigested Pronase (100 μg/ml for 1 h at 37°C); the nucleic acids were precipitated with ethanol, dissolved in 0.01× SSC (SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.4]), phenol extracted, re-precipitated with ethanol, redissolved in 0.01× SSC, and treated with pancreatic RNase A (20 μg/ml at 37°C for 1 h). After two further phenol extractions and one extraction with phenol-chloroform-isoamyl alcohol (50:49:1), the DNA was collected by ethanol precipitation and dissolved in 0.01× SSC. The Hirt pellet fraction was suspended in 0.05 M NaCl–0.001 M Tris-hydrochloride (pH 7.4) by homogenization in a Dounce homogenizer followed by passage through a 25-gauge hypodermic needle. The suspension was then treated with predigested Pronase (200 μg/ml for 2 h at 37°C), and the nucleic acids were precipitated with ethanol. The DNA was further purified as described above for the Hirt supernatant fraction. The total yield of DNA was approximately 1 mg/10^8 cells; of this, 1 to 12% (see Table 3) was found in the Hirt supernatant fraction, presumably due to random fragmentation of the high-molecular-weight chromosomal DNA during the lysis procedure.

For use in the reassociation experiments, the DNA was fragmented by sonic treatment to an average fragment size of 200,000 daltons (Raytheon 10-kc/s sonic vibrator operated at full power for 40 min), extracted once with phenol-chloroform-isoamyl alcohol and once with chloroform-isoamyl alcohol, precipitated with ethanol, resuspended in 0.01 × hybridization buffer (HB; HB is 0.5 M NaCl plus 0.02 M Tris-hydrochloride [pH 8.05]), and dialyzed exhaustively against 0.01× HB. The concentration of DNA was determined both from the OD_{260} (1 OD_{260} was taken as 50 μg of DNA per ml) and by the diphenylamine reaction (5).

In vitro labeling of SV40 DNA. SV40 DNA (component I) purified by equilibrium centrifugation in cesium chloride-ethidium bromide gradients followed by sedimentation in neutral sucrose gradients as described previously (17, 26) was labeled in vitro by the nick repair method, modified from the procedure of Maniatis et al. (20). The reaction mixture contained (in 100 μl) 30 mM Tris-hydrochloride (pH 7.9); 5 mM $MgCl_2$; 13 mM β-mercaptoethanol; 50 μg of bovine serum albumin per ml; 500 pmol each of dATP, dCTP, and dGTP; 500 pmol of [α-^{32}P]TTP (135 Ci/mmol); and 0.73 μg of SV40 DNA. The mixture was preincubated at 15°C for 10 min; the reaction was then initiated by adding 2 μl of a DNase solution (DNase I from Worthington Biochemicals Corp., Freehold, N.J.; 0.1 μg in water per ml) and 5 U of *Escherichia coli* DNA polymerase I (Boehringer Mannheim Corp., New York). After 1 h at 15°C, the reaction was terminated by dilution into 1 ml of 0.01 M Tris-hydrochloride (pH 8.0)–0.01 M EDTA–0.5% sodium dodecyl sulfate (SDS). The reaction contents were then extracted twice with phenol, the aqueous

phase was passed through a Sephadex G-50 column equilibrated with the dilution solution noted above, and the high-molecular-weight fractions were pooled. The specific activity of the SV40 DNA, at the time of labeling, was 6.4×10^7 cpm/μg; the size of the labeled DNA fragments was in the range of 7 to 16*S*.

DNA reassociation. The in vitro labeled SV40 [^{32}P]DNA (after sonic vibration as above) was mixed with excess sonically treated cellular DNAs, denatured by heating for 7 min at 110°C, quenched in liquid air, and brought to 0.5 M NaCl–0.02 M Tris-hydrochloride (pH 8.05). Aliquots (100 μl) of the mixture were then sealed in Pyrex capillaries and incubated at 69.5°C. After various times of incubation, capillaries were withdrawn and the degree of reassociation of the labeled SV40 DNA was assayed by resistance to S1 nuclease, as described previously (12).

Preparation of SV40 ^{3}H-labeled cRNA and DNA-RNA hybridization procedure. RNA complementary (cRNA) to plaque-purified SV40 DNA was prepared in a 0.8-ml reaction mixture containing 20 μg of SV40 DNA I; 100 μg of *E. coli* RNA polymerase (Sigma Chemical Co., St. Louis, Mo.); 0.05 M each ATP, CTP, and GTP; 0.15 M NaCl; 0.004 M $MgCl_2$; 0.001 M $MnCl_2$; 0.001 M 2-mercaptoethanol; 0.04 M Tris buffer (pH 7.9); and 1.8 mCi of [^{3}H]UTP (Schwarz/Mann, Div. of Becton, Dickinson & Co., Orangeburg, N.Y.; 49 Ci/mmol). Incubation was for 1 h at 37°C, and the reaction was terminated by the addition of 80 μg of RNase-free DNase (Worthington Biochemicals Corp.). After 15 min at 37°C, 1 mg of yeast tRNA and SDS to a concentration of 0.5% were added, and the RNA was further purified by phenol extraction, passage through Sephadex G-100, and alcohol precipitation.

DNA was immobilized on nitrocellulose membrane filters (14). For hybridization, each filter was incubated in 0.8 ml of a solution containing 3× SSC, 0.5% SDS, and 4×10^6 cpm of SV40 ^{3}H-labeled cRNA. After incubation at 68°C for 24 h, the filters were washed three times with 3× SSC at 60°C, treated with RNase (Worthington Biochemicals Corp.; 20 μg/ml in 2× SSC for 30 min at 37°C), rewashed with 3× SSC at 60°C under suction, dried, and counted.

RESULTS

Isolation of transformed clones susceptible to superinfection. The African green monkey cell lines CV1 and BSC-1 are highly susceptible to lytic infection with SV40. To minimize the cell-killing effects of the virus and the consequent selection of nonpermissive transformants, the monkey cells were infected with UV-irradiated virus and the transformed colonies were isolated in soft agar containing anti-SV40 antiserum (see above for full details of the procedure). The efficiency of transformation, as measured by colony formation in soft agar, was 0.001% (20 colonies out of 2×10^6 infected cells). No colonies were observed when 2×10^6 uninfected CV1 or BSC-1 cells were similarly plated in soft agar.

To screen for susceptibility to superinfection, each population of cells, derived from a single colony growing in soft agar, was infected with SV40 at an input multiplicity of ~50 PFU/cell and observed for cytopathic effects (CPE). Out of 11 SV40-transformed CV1 clones, 5 responded with complete CPE, 5 displayed partial CPE, and 1 clone responded with only marginal CPE. Out of 33 SV40-transformed BSC-1 clones, more than 50% responded with complete CPE upon superinfection.

The properties of three of the susceptible SV40-transformed populations of CV1 cells (designated as the C2, C6, and C11 lines) were studied in detail to determine whether they conformed to the accepted criteria for transformed cells. The properties examined (Table 1) were cell density inhibition, serum dependence as measured by cloning efficiency in 10 and 2% fetal calf serum, ability to grow on top of normal cell monolayers, colony formation in 0.33% agar, and stable expression of the SV40 T-antigen. The results are summarized in Table 1 and Fig. 1. It will be noted that as compared with normal CV1 cells, the transformed lines grew faster and reached higher levels of cell density per unit of surface area. The transformed cells also exhibited substantially increased levels of plating efficiency in 10 and 2% fetal calf serum and when seeded on top of normal cell monolayers. Their colony-forming ability in soft agar (the basis for the original selection procedure) was several orders of magnitude greater than that of normal cells. Finally, it will be noted that essentially all of the cells of the C2, C6, and C11 lines were T-antigen positive. The characteristic transformed cell growth properties and T-antigen expression have been stably maintained over many cell generations during the past 2 years.

Permissiveness of the transformed monkey lines. The ability of the SV40-transformed monkey lines to support the replication of superinfecting virus was measured in a variety of ways. Starting from a low multiplicity of infection, the yields of virus produced in the transformed cultures were found to be similar to those produced in normal CV1 plates (Fig. 2). To measure the proportion of infectible cells, the normal and transformed cell populations were infected at a high multiplicity, and the proportion of SV40 V-antigen-positive cells was determined at different times postinfection. Table 2 shows that essentially all of the transformed cells, like the untransformed CV1 population, became V-antigen positive by 37 h post-

TABLE 1. *Properties of SV40 UV-transformed monkey CV1 lines*

Cell line	Cell density (cells/cm²)[a]	Cloning efficiency (%)[b]			Colonies in 0.33% agar (%)[c]	T-antigen (%)[d]
		10% FCS	2% FCS	On BSC-1 monolayers		
Normal CV1	145,000	2	0	<0.05	<0.001	0
Transformed CV1						
C2	550,000	79	50	40	9	90
C6	390,000	82	52	25	1.4	90
C11	500,000	NT[e]	20	40	1.3	90

[a] A total of 2×10^5 cells were seeded into 6-cm-diameter plates (in medium with 10% calf serum). After 9 days of incubation, the cells were harvested and counted. The results are expressed as the number of cells per square centimeter of surface area.

[b] A total of 200 cells were seeded into 9-cm-diameter plates (in medium with either 10 or 2% fetal calf serum [FCS] as indicated), incubated for 2.5 weeks, and then stained with a 10% Giemsa solution to facilitate colony counting. To determine the ability of the cells to grow on top of normal cell monolayers, portions of 100 and 1,000 cells were seeded on 3-day-old BSC-1 monolayers. The medium (10% FCS) was changed weekly; after 1 month, the cultures were stained with Giemsa solution, and the number of colonies was counted.

[c] Cells were seeded in soft (0.33%) agar by the method of Macpherson and Montagnier (19). The colonies of transformed cells were counted after 6 weeks of incubation.

[d] Measured by direct immunofluorescence, as described in the text.

[e] NT, Not tested.

infection. None of the transformed cells (approximately 100,000 were scored) displayed any V-antigen reactivity prior to SV40 superinfection.

The rate of SV40 DNA synthesis, after high-multiplicity infection, was measured as described in the legend to Fig. 3. It will be noted that the rate of virus DNA synthesis, determined during 2-h periods spread over 10 to 60 h postinfection, was somewhat slower in the transformed cells as compared with normal CV1 cells. Nevertheless, the overall accumulative yields (Figs. 2) and the high proportion of infectible cells (Table 2) show that each of the three SV40-transformed monkey lines fully supports the replication of superinfecting virus. An additional useful property is that the integrity of the transformed cell sheet is maintained under agar, and well-defined plaques appear after SV40 infection. Plaque size is slightly smaller than it is in normal CV1 monolayers, and the number of plaques induced by a standard SV40 stock was, after 18 days of incubation, 30 to 100% the number obtained on normal CV1 cell sheets.

Lack of infectious virus recovery. Due to the high sensitivity of the transformed monkey cell populations to superinfection, the presence of a rare ($<10^{-8}$) virus-releasing cell would be readily detectable. However, no spontaneous release of infectious virus has been observed during 2 years of subculture. Similarly, no plaques have appeared in several hundred transformed cell populations maintained under agar. Plating of large numbers (10^7 cells/ml) of sonically disrupted transformed cells on top of normal CV1 or BSC-1 monolayers has failed to release infectious virus. Cell fusion experiments between each of the transformed lines and normal CV1 or BSC-1 cells or between the transformed lines themselves (in double- and triple-fusion experiments) have also failed to produce infectious virus (B. Hoffman, unpublished data).

State and number of copies of the resident viral genome. DNA was isolated from normal and transformed cells by the Hirt lysis procedure, which distinguishes between high-molecular-weight chromosomal DNA and low-molecular-weight DNA, such as free viral DNA (15). The presence and quantity of viral sequences in each of the two DNA fractions were then measured by determining their ability to accelerate the reassociation of an SV40 DNA probe (13), radiolabeled in vitro to a high specific activity. The results, normalized to the diploid quantity of cellular DNA, are shown in Fig. 4 and Table 3. It will be noted that the bulk of the SV40 sequences in each of the transformed lines is associated with the high-molecular-weight chromosomal DNA, as defined by the fraction that is pelleted in the Hirt lysis procedure. Only a minor portion of SV40 sequences was detected in the Hirt supernatant fraction, and some of these sequences can be accounted for on the basis of fragmentation of the chromosomal DNA during the extraction procedure (Table 3). Specifically, in the case of the C2 and C6 lines,

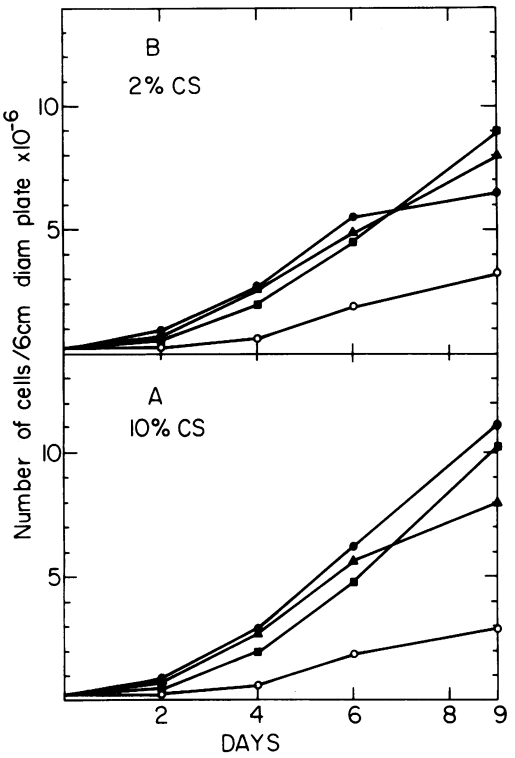

FIG. 1. *Growth curves of normal CV1 and SV40 UV-transformed CV1 lines. A total of 2 × 10⁵ normal and transformed cells were seeded in 6-cm-diameter plates in medium with 10% calf serum (A) or 2% calf serum (B). At days 2, 4, 6, and 9, the cells were harvested and counted. Symbols: ○, normal CV1 cells; ●, transformed C2 cells; ▲, transformed C6 cells; and ■, transformed C11 cells.*

only 2.8 and 4.4%, respectivley, of the total SV40 sequences detected were found in the Hirt supernatant fraction, and approximately 1% of the total chromosomal DNA was also found in this fraction (Table 3). In the case of the C11 line, about 15% of the total SV40 sequences were in the Hirt supernatant fraction, but 12% of the total chromosomal DNA was also present in this fraction.

The values shown in Table 3 for the average number of viral genome equivalents per transformed cell have been normalized to the diploid quantity of cellular DNA. Preliminary chromosome counts indicate that cells of the C6 and C11 lines contain, as does the CV1 line, a near diploid number of chromosomes, whereas the C2 line contains about twice that number. On this basis, the transformed monkey cells contain, on an average, from 1 to 2 viral genome equivalents per cell, the majority, if not all, of the viral sequences being associated with high-molecular-weight chromosomal DNA.

Can the transformed cells support the replication of SV40 tsA mutants at the restrictive temperature? Recent studies with nonpermissive cells transformed by SV40 carrying a temperature-sensitive mutation in gene *A* have shown that some of the transformed characteristics are lost when the cultures are grown at temperatures restrictive to the mutant virus (4, 16, 21, 24, 32). The gene *A* product is also known to be responsible for the initiation of SV40 DNA replication in permissive monkey cells (6, 31). Thus, even though SV40 DNA replication does not occur in nonpermissive cultures, the gene *A* product is expressed and appears to be involved in the maintenance of the transformed phenotype. Since the SV40 UV-transformed permissive monkey cells possess a stable transformed phenotype, we would expect a priori that the integrated viral genome contains a functional gene *A*. It was therefore of interest to determine whether the integrated viral genome can complement SV40 *tsA* mu-

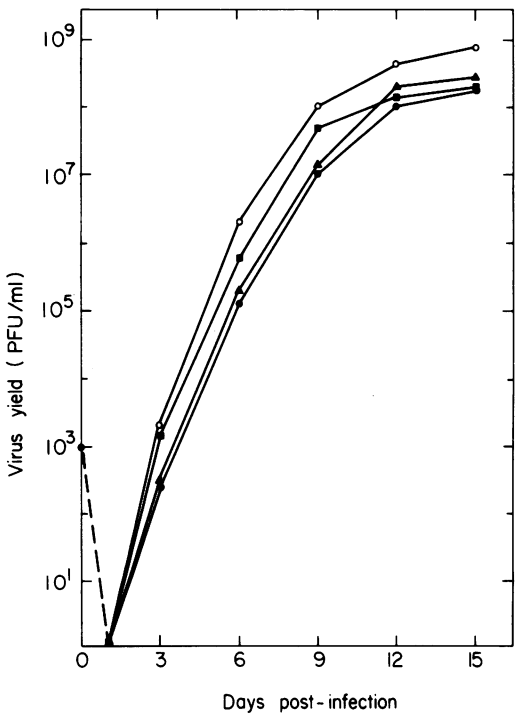

FIG. 2. *Virus yields in normal and transformed CV1 cells. Cultures of each cell line were infected with SV40 at a low multiplicity (10³ PFU/4 × 10⁶ to 10 × 10⁶ cells). At each time point, the total virus yield was harvested by freeze-thawing the culture three times and titered by plaque assay on BSC-1 monolayers. Symbols: ○, CV1 cells; ●, transformed C2 cells; ▲, transformed C6 cells; and ■, transformed C11 cells.*

TABLE 2. *V-antigen production in CV1 and SV40 UV-transformed derivatives infected with SV40*[a]

Cell line	V-antigen-positive cells (%) at h postinfection:				
	0	14	18	25	37
CV1	0	0	22	60	98
C2	0	0.03	6	42	97
C6	0	0.50	18	50	92
C11	0	0.10	10	36	96

[a] Cells of each line, growing on cover slips, were infected with SV40 at a multiplicity of 30 to 60 PFU/cell. At the indicated times, the cells were fixed and stained with fluorescent SV40 anti-V-antiserum, as described in the text. Approximately 200 microscope fields (each containing 100 to 150 cells) were scanned in the count of V-antigen-positive cells at the early time points (0 and 14 h postinfection).

tants at the restrictive temperature. The complementation effect would be expressed as the ability of *tsA* mutants to replicate at the restrictive temperature in transformed cells, but not in the nontransformed parental CV1 line.

To examine the above possibility, we infected transformed and nontransformed parental cultures with either wild-type (same virus used for transforming the CV1 line) or *tsA* SV40. The infected cultures were maintained at the permissive temperature (33°C) for 74 h, and then the rate of [^{3}H]TdR incorporation into viral DNA was measured after switching the cultures to the restrictive temperature (41°C). The results of such an experiment performed with SV40 *tsA30* (31) are shown in Table 4. It can be seen that none of the cell lines detectably supported the synthesis of *tsA30* DNA at 41°C, although replication of wild-type DNA was normal at this temperature. The same experiment repeated with two other SV40 *tsA* mutants, *tsA209* and *tsA239* (7), gave essentially identical results (data not shown). Control coinfection tests have shown that the wild-type SV40 strain 777 used for transforming the monkey cells will fully complement the growth of *tsA30* at 41°C. These tests have also established that SV40 strain 777 is a late *ts* mutant in that at 41°C it complements the growth of the *tsA*, but not the *tsB* or *tsC*, classes of mutants (Y. Gluzman, E. L. Kuff, and E. Winocour, manuscript in preparation).

The above experiments demonstrate that within the sensitivity of the method used, SV40-transformed CV1 cells do not appear to complement *tsA* mutants for viral DNA synthesis at the restrictive temperature. This implies that either the SV40-transformed CV1 cells do not synthesize active gene *A* products or that the products are made at levels too low to be detected in this type of experiment. In an attempt to increase the sensitivity of the assay, the SV40 *tsA*-infected cells were grown for 68 h at 33 or 41°C, after which the viral DNA was extracted by the procedure of Hirt (15) and immobilized on nitrocellulose membrane filters (14). These DNA filters were then hybridized with an excess of SV40 ^{3}H-labeled cRNA, synthesized in vitro by E. coli RNA polymerase using plaque-purified SV40 DNA as a template (see above). Under the conditions of hybridization used, the amount of RNA hybridized was proportional to the quantity of SV40 DNA present on the filter. The results of this experiment, together with the sensitivity calibration control, are shown in Table 5. After 68 h at 33°C, the four SV40 *tsA209*-infected lines accumu-

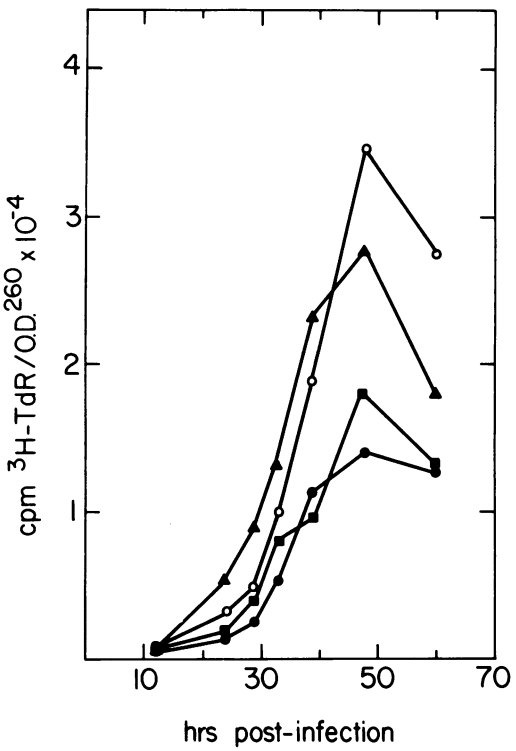

FIG. 3. *Rate of SV40 DNA replication in normal and transformed CV1 cells. Cultures of each cell line were infected with SV40 at a high multiplicity (50 to 100 PFU/cell). At each time point, the cells were labeled for 2 h with [^{3}H]TdR (2 μCi/ml; 15 Ci/mmol) and lysed, and the incorporation of [^{3}H]TdR into virus DNA in the Hirt supernatant fraction was measured, as described in the text. Variations in the number of cells per culture are corrected for by normalizing the results to the amount of OD_{260}-absorbing material in the Hirt pellet fraction (see text). Symbols: ○, CV1 cells; ●, transformed C2 cells; ▲, transformed C6 cells; and ■, transformed C11 cells.*

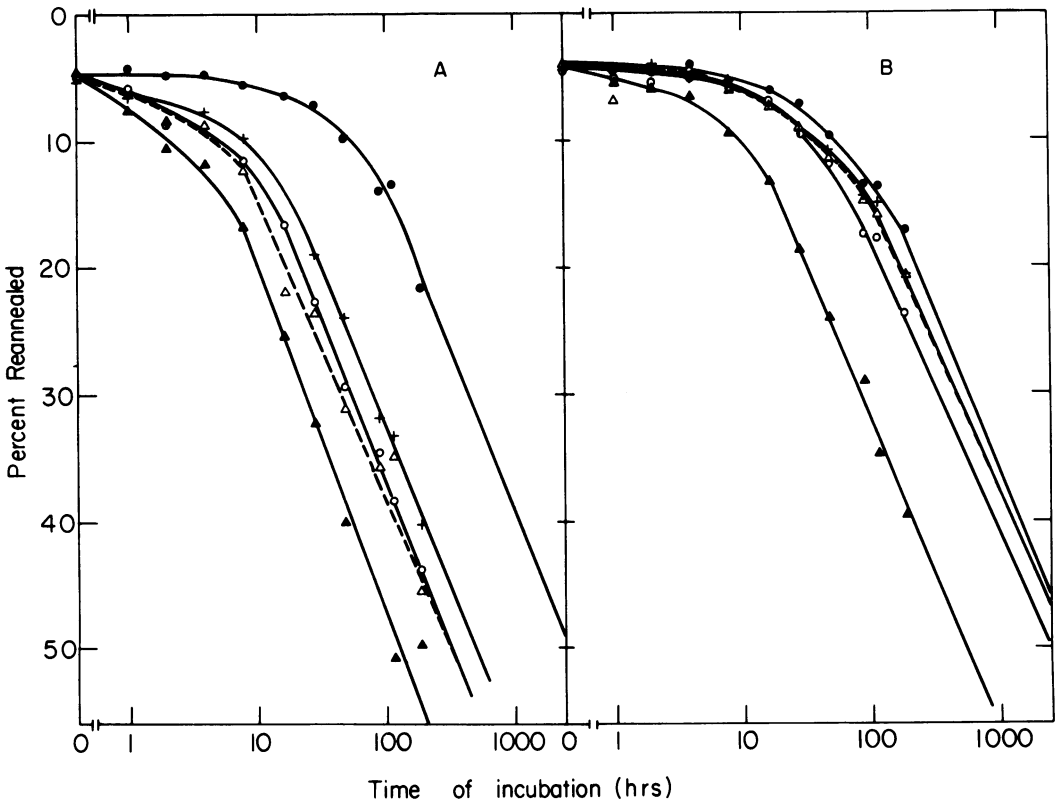

FIG. 4. *Reassociation of ^{32}P-labeled SV40 DNA in the presence of normal CV1 and transformed cellular DNAs. The in vitro synthesis of the ^{32}P-labeled SV40 DNA probe and the conditions of hybridization are given in the text. Each reaction contained 1,250 cpm of ^{32}P-labeled SV40 DNA (0.244 ng/ml) and excess unlabeled cellular DNA from the Hirt pellet fraction (1.3 to 2.0 mg/ml) (A) or the Hirt supernatant fraction (9 to 100 μg/ml) (B). See the text for details of the Hirt fractionation procedure. Reassociation was monitored by resistance to S1 nuclease, as described elsewhere (12). The unlabeled DNAs were derived from normal CV1 cells (●) and the transformed lines C6 (+), C11 (○), and C2 (△). The reconstruction reaction (▲) contained a mixture of unlabeled CV1 and SV40 DNAs in a ratio equivalent to 2.96 viral genomes per diploid cell (A) and 1.75 viral genomes per diploid cell (B). The extrapolated part (>20% reassociated) of the normal CV1 curve (●) was obtained by drawing the line parallel to that of the reconstruction curve (▲).*

lated 0.5 to 1.0 μg of SV40 DNA. In contrast, after 68 h at the restrictive temperature (41°C), less than 0.001 μg of SV40 DNA was present, irrespective of whether the transformed or parental nontransformed cell lines were used. Thus, less than 0.1% of the normal amount of DNA replication took place in the *tsA209*-infected, SV40-transformed lines at 41°C. It may also be seen in Table 5 that there was less SV40 *tsA209* DNA present after 68 h at 41°C than after only 6 h at 37°C. This finding may result from the elution of adsorbed *tsA209* virions that have failed to penetrate or from the degradation of parental *tsA209* DNA molecules within the cell.

The conclusion that the gene *A* product responsible for the initiation of viral DNA synthesis is not demonstratable in the three SV40-transformed, permissive monkey lines is in apparent conflict with the observation that the gene *A* product is required for the maintenance of the transformed phenotype in nonpermissive systems. Possible explanations for this discrepancy will be considered below.

DISCUSSION

The isolation of permissive SV40-transformed monkey cells depends upon procedures that minimize the cell killing effects of the virus and the consequent selection pressure for nonpermissive cells. Previous studies have shown that, compared with the plaque-forming ability, the transforming capacity of both polyoma virus (1) and SV40 (9) is relatively resistant to UV irradiation. By using SV40 irradiated with UV light at a dosage level that radi-

cally reduces the cytocidal effect without a comparable effect on the transforming capacity and by incorporating anti-SV40 antiserum into the soft-agar selection system to minimize reinfection, we have been able to isolate SV40-transformed monkey lines that fully support the growth of super-infecting virus. Indeed, the three transformed monkey lines studied in detail were comparable to untransformed CV1 monkey populations with respect to virus yields, proportion of infectible cells as measured by V-antigen synthesis, and ability to produce plaques under agar.

The permissive monkey transformed lines were initially selected by colony formation in soft agar (19), which appears to be a highly

TABLE 3. *Average number of SV40 genome equivalents in the transformed cells*[a]

Cells	Hirt pellet fraction		Hirt supernatant fraction	
	Concn of SV40 sequences[b] (ng/ml)	Equivalents[c]/diploid cell	Concn of SV40 sequences[b] (ng/ml)	Equivalents[c]/diploid cell
Transformed				
C2	2.02	1.28	0.059	0.037 (1.0)[d]
C6	1.06	1.02	0.055	0.047 (1.0)
C11	1.71	1.36	0.150	0.211 (12.4)
Normal CV1 + SV40 DNA (reconstruction)	4.36	3.73[e]	1.271	2.141[f]

[a] See text and legend to Fig. 4 for experimental details.

[b] Since the concentration of SV40 sequences in the normal CV1 DNA control reaction (Fig. 4) is that of the ^{32}P-labeled SV40 probe (0.224 ng/ml), the *total* concentration of SV40 sequences (^{32}P-labeled SV40 DNA probe plus SV40 sequences in the transformed-cell DNA sample) is given by 0.224 × factor of increased reassociation (13). Hence, the concentration of SV40 sequences in the transformed-cell DNA sample is 0.224 × (factor of increased reassociation − 1).

[c] By taking the size of the SV40 genome as 3.2×10^6 and that of the diploid monkey genome as 4×10^{12}, the number of viral genome equivalents per diploid cell is given by $(C \times 4)/(D \times 3.2)$, where C is the concentration of SV40 sequences in nanograms per milliliter, and D is the concentration of total cellular DNA (Hirt pellet plus supernatant fractions) in milligrams per milliliter. The number of equivalents was calculated separately for values of 10, 20, 30, 40, and 50% reassociation in Fig. 4 and then averaged.

[d] Numbers in parentheses indicate the percentages of total cellular DNA found in the Hirt supernatant fraction.

[e] On the basis of the ratio of normal CV1 to SV40 DNA in the reconstruction mixture, the expected value is 2.96.

[f] Expected value is 1.75.

TABLE 4. *SV40 tsA30 DNA synthesis in normal and transformed monkey cell lines*[a]

Cell line	Virus	[^{3}H]TdR incorporation (cpm) for 1 h at (°C):		Ratio of 41°C/33.5°C
		33.5	41	
CV1	SV40$^+$	6,518 (6,580)	7,072 (7,157)	1.080
CV1	SV40 *tsA30*	2,330 (2,392)	64 (141)	0.024
C2	SV40$^+$	8,594 (8,731)	9,103 (9,241)	1.050
C2	SV40 *tsA30*	5,864 (6,001)	53 (191)	0.009
C6	SV40$^+$	4,731 (4,928)	3,492 (3,707)	0.740
C6	SV40 *tsA30*	4,245 (4,442)	28 (243)	0.007
C11	SV40$^+$	10,395 (10,515)	9,087 (9,254)	0.870
C11	SV40 *tsA30*	2,203 (2,323)	−37 (136)	0.000

[a] The cultures (25-cm^2 flasks) were either infected (2 to 5 PFU/cell) with wild-type SV40 (SV40$^+$) or SV40 *tsA30* or mock infected. After 74 h at 33°C, half of the cultures were transferred to 41°C, whereas the other half remained at 33°C. All incubations were in a water bath to facilitate temperature control. At 1 h after the temperature shift, the cultures were labeled for 1 h at either 33 or 41°C with prewarmed medium containing [^{3}H]TdR at 3 μCi/ml (15 Ci/mmol; Radiochemical Centre, Amersham, United Kingdom). The cells were then washed and lysed, and the level of virus DNA synthesis was determined as described in the text. The numbers represent the counts per minute retained by the filter after 5% trichloroacetic acid precipitation of one-tenth of the Hirt supernatant fraction. The numbers in parentheses represent the values before subtraction of the mock-infected control for that cell line at the appropriate temperature. The wild-type SV40$^+$ in the above experiments is the same plaque-purified stock that was used to transform the monkey cells.

TABLE 5. *SV40 tsA209 DNA synthesis as measured by hybridization with SV40 ^{3}H-labeled cRNA*[a]

Incubation conditions	Cell line	^{3}H-labeled cRNA hybridized (cpm × 10^{-3})
6 h, 37°C	CV1	65
	C2	49
	C6	41
	C11	60
6 h, 37°C; 68 h, 33°C	CV1	1,200
	C2	581
	C6	935
	C11	1,000
6 h, 37°C; 68 h, 41°C	CV1	1.7
	C2	1.4
	C6	1.1
	C11	2.4
Mock infected	CV1	0.8
	C2	0.8
	C6	0.8
	C11	1.2
Reconstruction expt: SV40 DNA/filter		
1 μg		1,000
0.1 μg		102
0.01 μg		10.5
0.001 μg		2.6
Blank filter		0.5

[a] Cultures of the appropriate cell line were infected (2 to 5 PFU/cell) with SV40 *tsA209* and incubated at 37°C for 6 h. The medium was then changed, and one culture of each cell line was either lysed immediately or incubated for 68 h at 33 or 41°C prior to lysis. Viral DNA was extracted by the Hirt procedure and immobilized on nitrocellulose membrane filters (see text). One-fifth of the total nucleic acid content in the Hirt supernatant fraction derived from a single culture was loaded onto a single filter that was then hybridized with 4×10^6 cpm of SV40 ^{3}H-labeled cRNA, as described in the text. The numbers in column 3 show the amounts of ^{3}H-labeled cRNA retained by the filters after RNase treatment. The numbers given for the reconstruction experiment show the results of a hybridization calibration curve obtained by immobilizing the indicated amounts of plaque-purified SV40 DNA on filters and hybridizing it against the same ^{3}H-labeled cRNA under identical conditions to those noted above.

selective and stringent transformation procedure (25). The growth properties of the permissive C2, C6, and C11 lines were found to be typical of those described for transformed cells derived from nonpermissive host species. Furthermore, these growth properties, like the stable expression of the SV40 T-antigen, have been maintained over many cell generations during the past 2 years. These results are in contrast to those reported recently by Noonan et al. (23), who observed that primary African green monkey kidney cells that survived infection with SV40 gave rise to T-antigen-positive established lines that did not exhibit the characteristic growth properties of transformed cells. These latter results may have been obtained because the lines were not initially selected for in soft agar and/or nonirradiated virus and primary cell cultures were used.

No instances of spontaneous endogenous virus production have been detected in the C2, C6, and C11 lines, nor has infectious virus even been recovered by cell fusion. By C_0t analysis, the number of viral genomes per transformed cell is small, on an average, no more than 1 to 2 viral genome equivalents. We do not, at present, know whether the 1 to 2 viral genome equivalents represent intact genomes or an unequal collection of subgenomic fragments, as has been reported for some SV40-transformed nonpermissive species (28). Most of the SV40 sequences in the transformed cells were confined to the high-molecular-weight DNA fraction that pellets in the Hirt lysis procedure. Only minor proportions (2.8, 4.4, and 15%, respectively, for the C2, C6, and C11 populations) of the total SV40 DNA sequences detected were found in the Hirt supernatant fraction. By the diphenylamine reaction, a small and variable proportion of total cellular DNA was also found in the Hirt supernatant fraction. Hence, the significance of the minor proportion of SV40 sequences detected in this fraction is difficult to assess, and their presence may simply be due to random fragmentation of chromosomal DNA during the extraction procedure. Since the vast majority (85 to 97%) of the SV40 sequences are associated with the high-molecular-weight DNA isolated from the transformed cells, we suggest that the viral genome is chromosomally integrated in such cells.

Studies with temperature-sensitive mutants of SV40 have indicated that the gene A product is responsible for both the initiation of viral DNA synthesis in permissive cells (6, 31) and the maintenance of the transformed phenotype in nonpermissive species (4, 16, 21, 24, 32). We assume that the SV40 gene *A* product is also responsible for the transformed growth properties of the three permissive monkey lines described herein. Although we have no evidence for this assumption, it should be recalled that the SV40 T-antigen is stably expressed in these transformed cells. Furthermore, the T-antigens

isolated from transformed, superinfected transformed, and lytically infected monkey cells were found to be of the same size, as measured by polyacrylamide gel electrophoresis (C. Prives, Y. Gluzman, and E. Winocour, manuscript in preparation). Why then does the gene *A* product in the transformed permissive monkey cells fail to complement the growth of superinfecting SV40 *tsA* mutants at the restrictive temperature? Two explanations may be considered for this result. First, the gene *A* product may be responsible for the transformed state of the monkey cells, but may be present in levels too low to be detected by the complementation techniques used. The experimental results given in Table 5, however, indicate that the assay was sufficiently sensitive to detect complementation at the level of 0.1% of the normal *tsA* DNA yield. It should also be noted that the transformed monkey cells synthesize substantial amounts of T-antigen; by complement fixation, the level of T-antigen synthesis in the transformed cells was 25% of that found in lytically infected monkey cells at the time of maximum virus DNA synthesis (unpublished data).

The second possibility is that the gene *A* product is pleiotropic in the sense that it has separate functions (which may be related or unrelated) for the maintenance of transformation and the initiation of SV40 DNA replication, and only the former function is expressed in transformed cells. In this connection, it is relevant to note that although the induction of host DNA synthesis by SV40 and the initiation of viral DNA synthesis are probably both functions of the gene *A* product, these two effects are separable by temperature in certain *tsA* mutants (8). If we accept the principle of a pleiotropic gene *A* product, then it may be supposed that the resident SV40 genome in the transformed permissive monkey cells contains a mutation in gene *A* (probably induced by UV irradiation), which affects the initiation of viral DNA synthesis but not the induction and maintenance of the transformed state. Since the lack of *tsA* complementation was observed with each of the three transformed lines, the selection of transformed cells carrying the proposed mutated SV40 genome could not have occurred by chance. Conceivably, such a mutation is required for the transformed permissive cells to survive. Our inability to find SV40-transformed CV1 lines that express the function for initiation of SV40 DNA synthesis may be analogous to the situation in coliphage lambda, in which the expression of the genes for lambda DNA synthesis results in death of the lysogen due to replication of the integrated prophage DNA in situ (see reference 8a).

Mouse cells transformed by polyoma virus frequently retain the capacity to support, to some degree, the replication of superinfecting polyoma, and cultured cloned populations derived from a transplantable polyoma-induced parotid tumor in DBA/2 mice have been shown to be fully susceptible to challenge infection with polyoma virus (34). In view of the results obtained with the SV40 monkey system, it is interesting to note that although some polyoma host range mutants grow better on polyoma-transformed mouse cells (2), the enhanced growth does not appear to be due to direct complementation by a virus product expressed by the resident polyoma genome (3). We are currently attempting to isolate SV40 host range mutants to determine whether these, and other SV40 deletion mutants, can be complemented by any of the products of the resident genome in the SV40-transformed permissive monkey cells.

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