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Establishment of a line of human fetal glial cells that supports JC virus multiplication

(human astroglial cells/progressive multifocal leukoencephalopathy/continuous cell line/simian virus 40 origin of replication mutant)

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ABSTRACT **Primary cultures of human fetal brain cells were transfected with plasmid DNA pMK16, containing an origin-defective mutant of simian virus 40 (SV40). Several weeks after DNA treatment, proliferation of glial cells was evident in the culture, allowing passage of the cells at low split ratios. Initially, only 10% of the cells demonstrated nuclear fluorescence staining using a hamster tumor antibody to the SV40 T protein. By the sixth passage, however, 100% of the cells reacted positively to the same antibody. During these early passages, the cells designated SVG began growing very rapidly and acquired a homogenous morphology. Cell division required only low serum concentrations, was not contact-inhibited, and remained anchorage dependent. These characteristics of the SVG cells have been stable through 25 passages or ≈80 cell generations. The SV40 T protein is continuously produced in the cells and can direct the replication of DNA inserts in the pSV2 vector, determined by *in situ* hybridization using biotin-labeled DNA probes, which contains the SV40 replication origin. More importantly, SVG cells support the multiplication of the human papovavirus JCV at levels comparable to primary cultures of human fetal glial cells, producing infectious virus as early as 1 week after viral adsorption. Their brain-cell derivation has been established as astroglial, based on their reactivity with a monoclonal antibody to glial fibrillary acid protein and lack of activity with an anti-galactocerebroside antibody, which identifies oligodendroglial cells. The SVG cells represent a unique line of continuous rapidly growing human fetal astroglial cells that synthesizes a replication-proficient SV40 T protein. Their susceptibility to JC virus (JCV) infection obviates a host restriction barrier that limited JCV studies to primary cultures of human fetal brain and thus should allow for more detailed molecular studies of human brain cells and JCV that infects them.**

Progressive multifocal leukoencephalopathy (PML) is a demyelinating disease affecting the oligodendroglial cells of the human brain (1). The plaque-like lesions that develop in demyelinated areas contain papovavirus particles, which are characteristic of PML (2, 3). In 1971, Padgett and her colleagues (4) isolated a human papovavirus (JC virus; JCV) from PML brain tissue by inoculation of primary cultures of human fetal glial cells (PHFG). Although there have been many attempts since then to propagate the JCV in other species and types of cells (5–7), human fetal glial cultures remained the almost exclusive susceptible host for JCV growth. There are, however, several significant problems in using PHFG cells for JCV studies. It is often difficult to obtain human fetal brain and propagate cells in culture for the time necessary for JCV growth. Such cultures are also both quantitatively inconsistent in the number of glial cells that survive culturing and qualitatively inconsistent in their susceptibility to JCV infection. What could overcome this problem would be a continuous line of susceptible cells, eliminating the inherent difficulties associated with PHFG cultures.

One approach to the solution to such a problem has been infection of cells with oncogenic viruses that have the ability to transform cells into immortalized cultures. Recently, Miranda *et al.* (8) have demonstrated that homogenous populations of human myoblast cells could be isolated from skeletal muscle cells after transformation with simian tumor virus 40 (SV40). Santoli *et al.* (9) and Shein (10) have transformed human adult brain and fetal glial cells, respectively, using SV40. However, infectious SV40 was shed from these cells and, in the case of the fetal brain, the life span of the cells lasted only 13–39 cell passages. To avoid this problem, Boast *et al.* (11) and Major and Matsumura (12) used a DNA replication-defective mutant of SV40 to transform human fibroblast and kidney cells. The SV40 origin-defective mutants were developed by Gluzman *et al.* (13) and were used successfully to produce monkey cells able to genetically complement both lethal and conditional lethal mutants in the early region of the SV40 genome (14). We report here that an origin-defective mutant of SV40 is able to immortalize human fetal glial cells. These cells, SVG, provide rapidly growing cultures of human astroglial cells that are capable of producing infectious JCV.

MATERIALS AND METHODS

Cell Cultures. Human fetal brain material was dissected from 8- to 12-week-old abortuses, aspirated through a 19-gauge needle, washed twice in Eagle's minimal essential medium (EME medium) and planted into 25-cm^2 tissue culture flasks treated with poly-D-lysine (0.1 mg/ml for 5 min). The cultures were grown and maintained on EME medium supplemented with 20% fetal bovine serum/75 μg of streptomycin per ml/75 units of penicillin per ml/1% (vol/vol) dextrose/2 μg of fungizone per ml (GIBCO).

Virus and DNA Preparations. The Mad-1 strain of the human papovavirus JCV was acquired as a lyophilized stock from the American Type Culture Collection. Growth of the virus was preliminarily done in PHFG cultures. Virion production was assayed by hemagglutination (HA) of human type O erythrocytes. JCV genomic DNA was prepared from the plasmid pJC (15) purified after *Bam*HI and *Hha* I digestion, and passage over a Sephacryl S-1000 column (Pharmacia). The pSV2 vector was constructed by Mulligan and Berg (16) and distributed by Bethesda Research Laboratories. The pSV2-JC recombinant plasmid was constructed using conventional recombinant DNA techniques conforming to the current National Institutes of Health guidelines. The ori-

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Abbreviations: PML, progressive multifocal leukoencephalopathy; SV40, simian virus 40; GFAP, glial fibrillary acidic protein; galC, galactocerebroside; PHFG, primary human fetal glial; JCV, JC virus; HA, hemagglutination.

gin-defective mutant of SV40 (mutant 1-11) was provided by Y. Gluzman as an insert in the pMK16 vector at the *Bam*HI site. All plasmids were grown in *Escherichia coli* K-12 hosts, amplified using chloramphenicol, and purified by a cleared lysis method (17, 18).

Biotin Labeling of JCV DNA. JCV genomic DNA was labeled with biotinylated dUTP by nick-translation procedures described by Brigati *et al.* (19). Trace amounts of [^{3}H]dATP were used during incorporation to follow the efficiency of the nick-translation. The JC DNA probe contained ≈18% substitution of dTTP with biotin-dUTP. The JC-biotinylated probe was separated from unincorporated nucleotide triphosphates using a column of Sephadex G-50.

***In Situ* DNA·DNA Hybridization.** Cells that would undergo hybridization were planted directly onto glass coverslips treated with poly-(D-lysine) and placed in 15-cm^2 cluster dishes (Costar, Cambridge, MA). Virus infection or DNA transfection was done on cells after establishment of growth. After the appropriate time period, the coverslips were removed and fixed in 4% paraformaldehyde. The *in situ* hybridization protocol followed the outline of Brigati *et al.* (19) with some modifications. Briefly, coverslips were attached to microscope slides using epoxy, washed in phosphate-buffered saline, treated with 0.1 M HCl for 10 min, 0.01% Triton X-100, rinsed in phosphate-buffered saline, and digested with 300 ng of protease per ml for 10 min. The coverslips were rinsed three times in phosphate-buffered saline/glycine and refixed in 4% paraformaldehyde before dehydration in graded ethanol concentrations. The hybridization reaction mixture routinely contained 50% (vol/vol) deionized formamide/10% dextran sulfate/400 μg of herring sperm DNA per ml/20 μg of biotinylated probe DNA per ml in 2× NaCl/Cit (0.3 M NaCl/0.03 M Na citrate). Twenty microliters of the hybridization mixture was applied per coverslip. A larger coverslip was placed on top and sealed by rubber cement. The cells and probe DNA were denatured together in an oven set at 80°C for 15 min, cooled quickly to room temperature, and placed at 37°C for 36 hr. Detection of the hybridization signal was made using the avidin–biotin–peroxidase complex as described by Hsu *et al.* (20).

Fluorescent Antibody Assays for SV40 T Protein and Glial Cell Markers. Cells to be tested for the nuclear SV40 T protein and glial fibrillary acidic protein (GFAP) were fixed on coverslips using acetone/methanol treatment at −20°C for 10 min. Cells designated for GFAP staining were also fixed with 4% paraformaldehyde. The anti-SV40 T antiserum was derived from a tumor-bearing hamster described previously (21). The anti-GFAP antibody was acquired from Lab Systems (Helsinki, Finland) as a mouse monoclonal antibody made against the human GFAP of an astrocytoma. The mouse anti-galactocerebroside (galC) was a gift from D. van Alstyne. Cells to be tested with anti-galC antibody were harvested live from culture, washed in EME medium and Hepes buffer, and allowed to react with sera in Microfuge tubes.

RESULTS

Development of SV40-Transformed Human Glial Cells. Primary human fetal brain tissue between 8 and 12 weeks of gestation can be planted into cell cultures (22) and, if refed at weekly intervals, will survive for several months but show little cell proliferation. Since glial cells, however, do have mitotic capability, we thought it possible to extend their life span in culture by transfecting the cells with a mutant of SV40 DNA. This DNA is itself unable to multiply because of a deletion in its origin of replication (ori$^-$), but it is able to transform cells to unlimited growth potential (14). PHFG cultures were grown in 25-cm^2 flasks for 3 weeks and then transfected with 100 μg of plasmid DNA (pMK16) per flask containing the SV40 ori$^-$ mutant 1-11, using the calcium phosphate precipitation technique (23). For the first 3–4 weeks, the cultures appeared normal but required weekly refeeding. After this time, proliferation of glial cells in separate areas of the flasks was evident. The cells were transferred by trypsinization (0.025%) to new cultures at a 1:2 ratio and were designated SVG. Fluorescent antibody assays to detect the SV40 T protein using a hamster tumor-bearing serum demonstrated that only 10% of cells were positive. The cultures were passaged every 10 days before any increase in number of T-protein-positive cells was detected. By 14 weeks, or the 6th passage up to the current 25th passage, 100% of cells in all cultures showed nuclear staining with the anti-SV40 T-protein antibody. Radioimmunoprecipitation assay had also identified the 94-kDa SV40 T protein resolved on polyacrylamide gels (data not shown). Table 1 lists the biological properties of the SVG cell line that have been consistent for all passages tested to date. The SVG cells display the phenotypes of a continuous cell line because they grow to very high saturation density with an 18-hr generation time. They do not show the transformed phenotype of anchorage-independent growth, however, which is characteristic of SV40-transformed cells. The cell morphology also was not altered during the course of establishment of the cell line. There was never a dense piling up or foci of cells formed. There was a transition from nonhomogeneity of cell types including astrocytes, oligodendrocytes, and fibroblast-type cells into a homogenous cell morphology more typical of the astroglial cell.

Identification of SVG Cells as Astroglial. Characteristically, astroglial cells can be recognized by the presence of an intermediate filament composed of GFAP. Oligodendroglial cells, on the other hand, are myelin-producing cells and can be identified by their synthesis of a galactocerebroside, galC, which is a component of myelin. We tested the SVG cells in comparison to PHFG cells for the presence of the neural cell markers. Fig. 1 (*A–D*) shows photomicrographs of normal PHFG cells as follows: (*A*) oligodendroglial cells that grow in clumps and send out thin processes and astroglial cells that surround the oligodendroglial cells and are recognized by their larger nuclei; (*B*) reactivity of the identical cultures shown in *A* to antibody to galC detecting only the cells in the dense clumped area; (*C*) reactivity of the same culture shown in *A* with antibody to GFAP detecting the astroglial cells present surrounding the oligodendroglial cells; and (*D*) no reactivity of PHFG cells to antibody to SV40 T protein. Fig. 1 (*E–H*) shows photomicrographs of SVG cells at their 15th passage as follows: (*E*) a homogenous morphology of cells similar to astroglial cells; (*F*) no reactivity to anti-galC antibody; (*G*) reactivity to anti-GFAP antibody, which stains the cytoplasm in a pattern similar to the staining of SVG cells for actin fibers, as typical of transformed cells (data not shown); (*H*) 100% of cells reacting to anti-SV40 T-protein antibody.

Mutiplication of JCV in SVG Cells. To assess whether SVG cells could support JCV multiplication in a direct comparison with PHFG cells, we conducted the following experiment. Human fetal brain tissue was uniformly distributed onto coverslips in multiple 15-cm^2 cluster dishes and was

Table 1. Biological characteristics of the SVG cell line

Cell type	Anchorage independence	Saturation density, cells per cm^2	% T protein
PHFG	—	1×10^4	<0.001
SVG	—	2×10^5	100

Assays for anchorage-independent growth were done using soft agar colony growth (0.33%) on SVG cells at passages 10 and 15. Saturation density was determined by hemocytometer counts of cells trypsinized from confluent cultures in triplicate at cell passages 10 and 15.

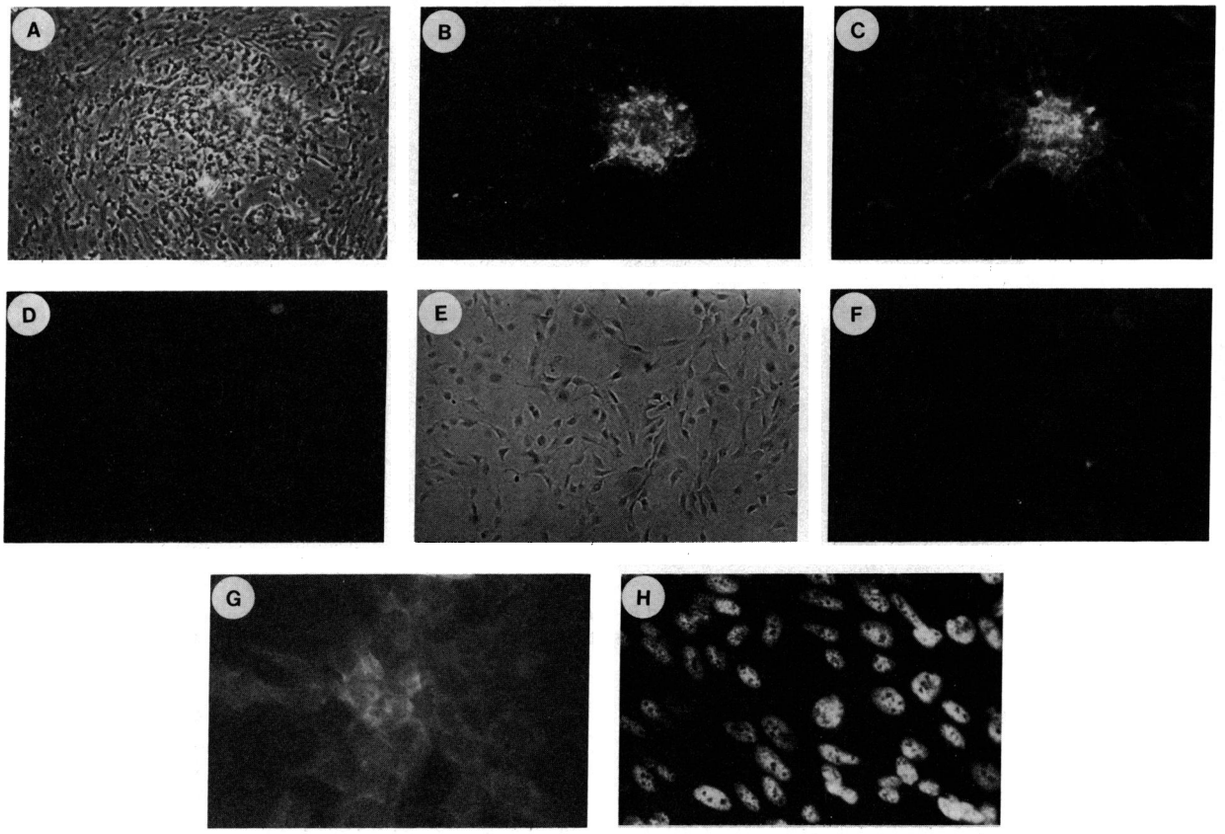

FIG. 1. Identification of cell types in cultures of PHFG (*A–D*) and SVG (*E–H*). (*A* and *E*) Representative field of cells in phase contrast, bright field; (*B* and *F*) use of mouse anti-galC antibody and rhodamine-conjugated goat anti-mouse antibody to detect oligodendroglial cells; (*C* and *G*) use of mouse anti-GFAP antibody and fluorescein-conjugated goat anti-mouse antibody to detect astroglial cells; (*D* and *H*) use of hamster anti-SV40 T protein and fluorescein-conjugated goat anti-hamster antibody. Note that cells in *A*, *B*, and *C* are identical, having been treated with anti-galC and subsequently with anti-GFAP antibodies and photographed using barrier filters N2 for rhodamine (galC) or H3 for fluorescein (GFAP).

allowed to establish almost confluent cultures of glial cells. SVG cells were plated into cluster dishes at low density so that time in culture before virus adsorption was similar to that of human fetal glial cells. JCV Mad-1 strain (160 HA units) was inoculated equally into each of six wells per cluster plate of SVG or PHFG cells. One week, 2 weeks, and 3 weeks after viral adsorption, coverslips were removed and fixed appropriately or cells were harvested for virus assays to examine the rate of JCV T-protein synthesis, DNA replication, and the amount of virus produced. Table 2 lists the data from this experiment. It should be noted that synthesis of the JCV T protein could not be evaluated in SVG cells because both JCV T and SV40 T proteins antigenically cross-react. The only available monoclonal antibody to JCV T protein is PAb108, developed by E. Gurney for SV40 T protein, which we found to cross-react with the JCV T protein (21).

In the PHFG cells, JCV T protein was detected in the majority of cells (70% and 50%) in the first 2 weeks. By the end of the 3rd week of infection, the number of cells producing T protein was decreased to 15%, probably reflecting loss of infected cells to cytolysis because of JCV production. The decrease in number of cells with detectable T protein may also reflect the autoregulation of T-protein synthesis, as replication and late gene transcription begin as demonstrated for the SV40 life cycle (24).

Viral DNA replication was assayed by *in situ* DNA–DNA hybridization of infected cells with a biotin-labeled JCV DNA probe. We chose this technique because we wanted to quantitate the percentage of cells replicating JCV DNA while maintaining the morphology of oligodendroglial and astroglial cells. This nonradioisotope technique is clearly less sensitive in detecting low copy numbers of viral DNA, but it is superior to the use of isotopically labeled probes in the preservation of cell structure, as demonstrated by Singer and Ward (25), and it allowed us to accurately assess the

Table 2. Kinetic study of JCV multiplication in SVG cells and PHFG cells

	7 days*			14 days*			21 days*		
	% T protein	% DNA	Virions	% T protein	% DNA	Virions	% T protein	% DNA	Virions
PHFG	70	6.2	20	50	9.2	640	15	35	10,240
SVG	NA	4.8	≈10	NA	33	320	NA	53	5,120

*Time period after virus adsorption. T protein was determined as percentage of cells staining by immunofluorescence assay. DNA replication was determined as percentage of cells hybridizing to biotin-labeled JCV DNA probe averaged from three independent experiments. Virions were detected by HA of human type O erythrocytes derived from infected cells harvested from the cell culture medium. NA, not applicable.

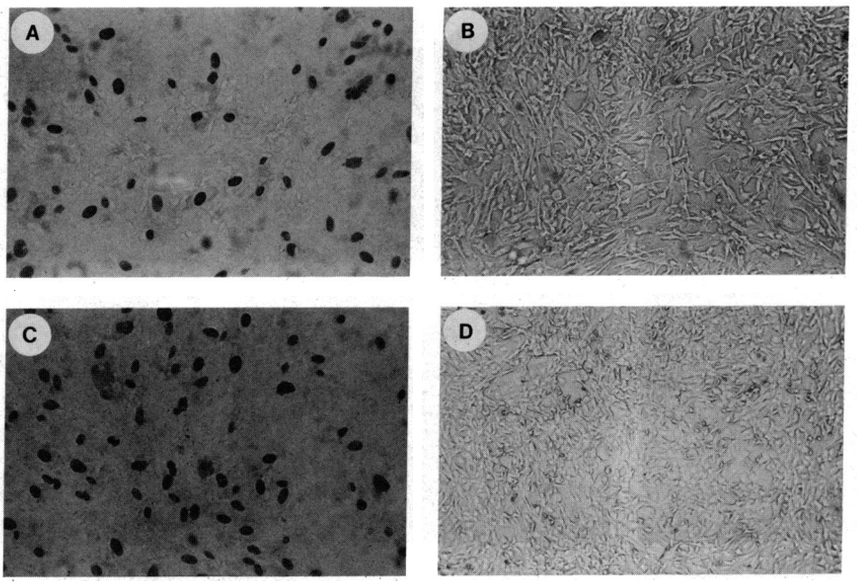

FIG. 2. *In situ* hybridization of JCV-infected PHFG cells (*A* and *B*) and SVG cells (*C* and *D*) with a JCV biotin-labeled DNA probe (*A* and *C*) or a control with no probe DNA (*B* and *D*). Darkly stained nuclei result from biotin–avidin–peroxidase complex reacting with biotin-labeled DNA in the presence of H_2O_2 and diaminobenzidene.

percentage of cells replicating JCV DNA. Data are summarized in Table 2 and are shown in Fig. 2. PHFG cultures showed 6.2% of cells synthesizing viral DNA during the first 2 weeks of infection. Between the 2nd and 3rd weeks, at the time when few cells were synthesizing T protein, there was a 4-fold increase in the number of cells replicating JCV DNA. In SVG cells, the increase in the number of cells hybridizing to JCV DNA probe between the 1st and 2nd weeks was 7-fold, and between the 2nd and 3rd weeks it was 1.6-fold. The striking difference between JCV DNA synthesis in PHFG cells and SVG cells was at the end of the 2nd week, at which time only 9.2% of PHFG cells compared to 33% of SVG cells hybridized to JCV DNA probe (Fig. 2).

Virion production was measured by HA of human type O erythrocytes. Interestingly, JCV was produced in both cell types at the same rate, a 32-fold increase between the 1st and 2nd weeks and a 16-fold increase between the 2nd and 3rd weeks. Infectious JCV, recovered from both cell types, grew in cultures of either PHFG or SVG cells. The 2-fold difference in HA titer of JCV produced in the PHFG cells compared to SVG cells (Table 2) was not considered significant because of the limitations of sensitivity of this assay.

T Protein in SVG Cells and DNA Replication. The SV40 T protein produced in cells transformed by SV40 ori$^-$ mutants is capable of directing the replication of SV40 DNA if it contains an intact origin of replication (13, 14). We wanted to test the SV40 T protein made in SVG cells for this same ability by using an *in situ* hybridization assay and the JCV DNA biotin-labeled probe. Therefore, we inserted the entire JCV DNA genome into the *Bam*HI site of the plasmid vector pSV2. *Bam*HI endonuclease cleaves the JCV DNA a single time in the middle of the coding sequence for the JCV T protein, thus preventing its synthesis. The pSV2-JC plasmid was then transfected into SVG and SV1 cells at 15 μg/15-cm^2 cluster plate. SV1 cells, a line of human embryonic kidney cells established by the same SV40 ori$^-$ mutant DNA as SVG cells, produces a T protein that functions for replication (12). JCV DNA made linear by *Bam*HI digestion was

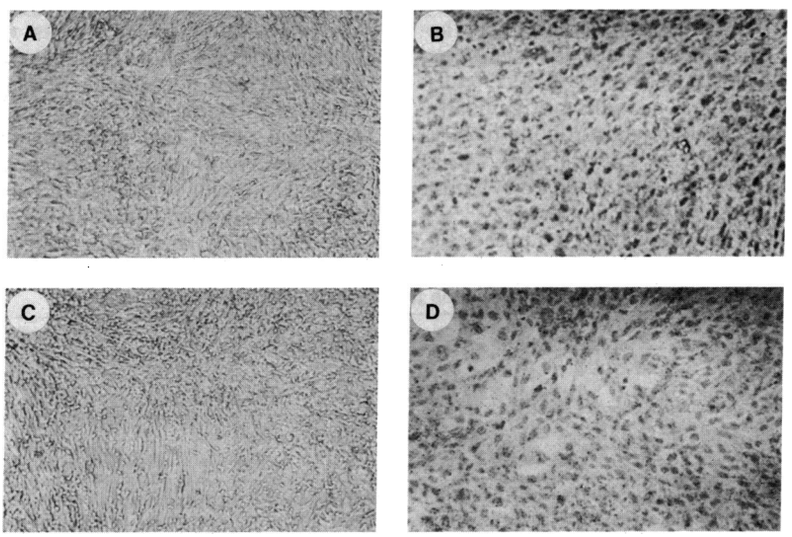

FIG. 3. *In situ* hybridization of SVG cells (*A* and *B*) and SV1 cells (*C* and *D*) with a biotin-labeled JCV DNA probe at 16 hr (*A* and *C*) and 110 hr (*B* and *D*) after transfection with the pSV2-JC plasmid DNA. Detection of hybridization signal is described in Fig. 2 legend.

transfected into separate cultures of SVG and SV1 cells. The cultures were then hybridized to the JCV DNA biotin probe 16 hr after transfection. The photomicrographs in Fig. 3 show the results of this experiment. No hybridization signal was detected at 16 hr after transfection, eliminating the possibility that a positive hybridization signal could result from hybrids forming between the input, transfected DNA, and the probe DNA. However, with sufficient time for replication to proceed, at 110 hr, 80% of both cell types gave positive hybridization signals. The linear JCV-DNA-transfected cells were not positive in the hybridization assay at 110 hr (data not shown). This indicated that JCV DNA cleaved in the middle of its T-protein coding sequence, as presented in the pSV2-JC vector also, cannot replicate its DNA within the time examined by this experiment. Therefore, the T protein in SVG cells must be able to recognize the SV40 origin sequences in the plasmid and direct the replication of the entire pSV2-JC molecule.

DISCUSSION

The experiments reported here describe a unique cell line of human fetal glial cells established by introduction of the ori$^-$ mutant of SV40. Their growth patterns (Table 1) define the cells as a continuous culture. Their reactivity to anti-GFAP antibody and cell morphology (Fig. 1) identify the cells as astroglial. The homogenous population of SVG cells would make them a good source of human fetal astroglial cells for more intense molecular and genetic experiments, which are not possible with mixed cell cultures derived from normal brain tissue.

In addition to these properties, the data presented in Table 2 and Fig. 2 demonstrate that SVG cells are able to support the multiplication of JCV. JCV is produced very slowly in PHFG cultures (26), requiring weeks of culturing during which time some virus can be harvested from cell culture fluid. The SVG cells, although growing rapidly, did not decrease the length of time JCV requires for multiplication throughout a culture. Infected SVG cells produced JCV at the same rate as the PHFG cells. Since SVG cells are astroglial, however, and JCV was thought to grow only in spongioblasts or oligodendroglial cells (27, 28), the data from these experiments provide direct evidence of JC virion production from astroglial cells.

The presence of the SV40 T protein in these cells and its effect on JCV production need to be studied further. The increase in the number of SVG cells replicating JCV DNA compared to PHFG by 2 weeks after infection may not be explained by a cooperative effect of the replication function for SV40 T protein. In previous experiments, JCV DNA was not replicated in SV1 cells even though the SV40 T protein was able to replicate a subgenomic fragment of BKV DNA containing its own origin of replication (12). Furthermore, Frisque has reported that SV40 T protein may bind JCV DNA sequences that are homologous to SV40 DNA binding sites (29), but his studies indicated, by examination of nucleotide sequences, that a third binding site for SV40 T protein may not be present in JCV DNA. These data, taken together, lead to a first approximation analysis that JCV DNA cannot effectively use the SV40 T protein to replicate its DNA and proceed to virion formation. A more detailed physical examination of the association between the SV40 T protein and JCV DNA remains to be done to answer this point directly.

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