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Transformation of Primary Hamster Brain Cells with JC Virus and Its DNA

R. J. FRISQUE,[1]* D. B. RIFKIN,[2] AND D. L. WALKER[3]

Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724[1]; Department of Cell Biology, New York University Medical School, New York, New York 10016[2]; and Department of Medical Microbiology, University of Wisconsin Medical School, Madison, Wisconsin 53706[3]

We transformed primary hamster brain cells with four isolates of JC virus and JC virus DNA. Several properties of these transformants were characterized and compared to those of simian virus 40 transformants isolated under identical conditions.

JC virus (JCV) is a human polyomavirus which is widely distributed in the human population (17). It is suspected of being the causative agent of progressive multifocal leukoencephalopathy, a demyelinating disease in which some of the infected cells become indistinguishable from malignant astrocytes of pleomorphic glioblastomas (25). When inoculated into hamsters, JCV produces a high incidence of a variety of tumors. Unlike the simian polyomavirus, simian virus 40 (SV40), which routinely induces papillary ependymomas upon intracranial inoculation of hamsters (8), JCV usually produces medulloblastomas, glioblastomas, or unclassified primitive tumors (25). In addition, JCV is the first virus shown to cause neuroblastomas (24) and pineocytomas (18) in hamsters and the only human virus known to cause brain tumors in a primate (13).

Despite the efficient tumor-inducing capacity of JCV, there have been no reports of this virus transforming cells in tissue culture. This report describes the first successful attempt to obtain such transformants.

Because the brain appears to be a preferred site for tumor formation by JCV, primary hamster brain (PHB) cells isolated from newborn animals were used in these studies. After one passage in culture, 10^6 cells were infected with either 1 μg of JCV or SV40 component I DNA, 10^6 PFU ($\sim 10^6$ fluorescent cell units; 1) of SV40, or 10^6 fluorescent cell units of JCV (a plaque assay is not available; 16). Four isolates of JCV (designated Mad 1, 2, 3, and 4), which were recovered from different PML patients, were used. At 1 to 3 days postinfection, cells were fixed in acetone and stained for immunofluorescent studies using serum from animals bearing SV40- or JCV-induced tumors. Approximately 1% of the SV40-infected cells and only about 0.01 to 0.1% of the JCV-infected cells contained T antigen. Because of earlier difficulties in demonstrating transformation with JCV, we chose the least stringent method for selecting viral transformants. Replica plates were repeatedly subcultured 1:2, and transformants were selected solely for their ability to survive repeated passage in culture. This procedure did not allow us to quantitate the frequency of transformation.

The cultures inoculated with SV40 or SV40 DNA were the first to show evidence of transformation (3 to 5 weeks, three to four passages). By this time cultures contained many giant cells, extensive cell death was evident, and colonies of rapidly dividing, morphologically altered cells had begun to appear. These cells were found to express T antigen(s).

PHB cultures infected with JCV DNA or the various JCV isolates were never found to exhibit the cytopathic effect. However, at 7 to 10 weeks postinfection (five to six passages), infected populations began demonstrating an altered appearance and an accelerated rate of growth. These populations also contained cells expressing viral T antigen(s).

By the eighth passage, >90% of the cells in both JCV- and SV40-infected cultures were positively stained with anti-T serum. Although homologous and heterologous antisera gave positive nuclear fluorescence in both types of cells, the homologous antiserum stained cells more intensely. The JCV transformants were primarily fibroblastic in morphology and exhibited a high level of mitotic activity even in confluent cultures. Except for appearing larger and somewhat less refractile, the JCV transformants were difficult to distinguish from SV40 transformants. Control cultures contained large, flat, irregularly shaped cells, and after five to six passages growth ceased. These cells remained negative for T antigen(s).

The phenotypes of the JCV-transformed PHB cells were characterized and compared to both normal PHB cells and SV40 transformants. All

transformants grew well in medium containing 1 or 10% fetal calf serum (Table 1). Although control cells grew in medium supplemented with 10% serum (doubling times increased dramatically with passage), they failed to grow in medium supplemented with 1% serum. Transformants also grew to higher saturation densities than PHB cells and exhibited dramatically increased cloning efficiencies. No significant differences were observed between JCV and SV40 transformants in the acquisition of these properties. A wide range of cloning efficiencies was observed when the transformants were plated in methylcellulose (Table 2), and the cloning efficiencies correlated with the acquisition of cellular tumorigenicity (as assayed by inoculation of weanling hamsters). Transformants which grew poorly in methylcellulose induced tumors less frequently or after a longer latency than lines which grew efficiently in semisolid medium. Only the cells transformed by Mad 1 DNA failed to follow this pattern (Table 2). Tumors induced by all cell lines rapidly progressed into large palpable masses. Imprints of sectioned tumors (cell transferred to a microscope slide by touching the tumor to the glass) were stained with the appropriate T or V antiserum, and the cells were shown to contain only T antigen. Sera from tumor-bearing animals were found to contain anti-T activity.

TABLE 1. *Growth properties of JCV- and SV40-transformed PHB cells*[a]

Cell line	Doubling time, 10% serum (h)	Doubling time, 1% serum (h)	Saturation density,[b] 10% serum	Cloning efficiency[c] (%)
PHB P1[d]	26	NG[e]	9.0×10^4	<1
PHB P3[d]	68	NG[e]	7.0×10^4	<1
SV40-PHB	20	26	3.2×10^5	63
C-SV40-PHB[f]	16	26	4.3×10^5	92
SV40 DNA-PHB[g]	23	37	3.4×10^5	99
Mad 1-PHB	24	29	1.4×10^5	49
C-Mad 1-PHB[f]	32	43	8.9×10^4	77
Mad 1 DNA-PHB[g]	25	52	2.4×10^5	69
Mad 2 PHB	28	34	1.1×10^5	47
Mad 3-PHB	22	51	2.2×10^5	74
HJC-15[h]	21	24	2.1×10^5	100

[a] Doubling times, saturation densities, and cloning efficiencies were carried out as described by Sleigh et al. (22). Cells were cultured in Hams F12 medium supplemented with 7 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid and 1 or 10% fetal calf serum.

[b] Units are the number of cells per square centimeter.

[c] Number of colonies of cells per 100 cells plated × 100.

[d] PHB cells after one or three passages in culture.

[e] NG, No growth.

[f] C-SV40-PHB and C-Mad 1-PHB are subcloned lines of SV40 and Mad 1 transformants (isolated separately from SV40- and Mad 1-PHB). Mad 1, 2, and 3 are different isolates of JCV.

[g] Transformants obtained after DNA transfection of PHB cells by the calcium technique (9).

[h] HJC-15 is a cell line derived from a JCV-induced tumor (25).

TABLE 2. *Phenotypes of JCV- and SV40-transformed PHB cells*

Cell line	PA[a] (%)	Loss of actin cables[b]	Growth in methylcellulose[c] (%)	Tumorigenicity[d] (avg latency in days)
PHB	0	−	<0.005	0/4
SV40-PHB	3	+	4.3	4/4 (18)
C-SV40-PHB[e]	28	+	<0.005	0/4
SV40 DNA-PHB[f]	11	+	0.1	3/4 (57)
Mad 1-PHB	18	±	0.005	2/4 (129)
C-Mad 1-PHB[e]	0	±	0.1	3/4 (105)
Mad 1 DNA-PHB[f]	0	−	<0.005	4/4 (128)
Mad 2-PHB	1	±	0.03	2/4 (99)
Mad 3-PHB	14	−	0.4	4/4 (31)
HJC-15[g]	21	−	14.9	3/3 (14)

[a] The assay for the secretion of plasminogen activator involved adding 50 μl of the medium in which the cells were maintained for 24 h to ^{125}I-labeled fibrin-coated plates and determining the amount of ^{125}I released after 90 min. The 100% level was set by solubilization with 25 μg of trypsin per ml.

[b] Immunofluorescently stained cells were screened for the presence (−), reduction (±), or loss (+) of actin cables.

[c] Cloning efficiency in methylcellulose was determined 3 weeks after plating 10^4 cells in 60-mm plates. Colonies >0.2 mm in diameter were counted.

[d] Weanling LHC Syrian hamsters were inoculated subcutaneously with 4×10^6 cells. The ratio is the number of animals with tumors/the number of animals injected.

[e] C-SV40-PHB and C-Mad 1-PHB are subcloned lines of SV40 and Mad 1 transformants (isolated separately from SV40- and Mad 1-PHB). Mad 1, 2, and 3 are different isolates of JCV.

[f] Transformants obtained after DNA transfection of PHB cells by the calcium technique (9).

[g] HJC-15 is a cell line derived from a JCV-induced tumor (25).

Two additional properties characteristic of transformed cells were also investigated. The secretion of plasminogen activator was detected using as an assay the digestion of ^{125}I-labeled fibrin as described by Rifkin and Pollack (20). Although some transformants resembled the parent cells in failing to produce plasminogen activator, several JCV- and SV40-transformed lines did produce significant levels of this protease (Table 2). The presence or absence of cytoplasmic actin cables was assayed by indirect immunofluorescence using rabbit antiactin antibody. SV40 transformants were found to have lost actin networks while the JCV transformants either partially or fully retained the pattern observed in the untransformed parent cell (Table 2). This retention of actin networks, along with a flatter cellular morphology, is the only significant difference that we have observed between JCV and SV40 transformants.

Three different procedures were used in an attempt to recover virus or viral DNA from transformed cells. Overlaying permissive cells with lysates of disrupted SV40 or JCV transformants (25) or cocultivating permissive and transformed cells (4) did not result in the expression of viral antigens or cytopathic effect, suggesting that spontaneous release of these viruses did not occur. However, viral cytopathic effect and SV40 DNA were detected after polyethylene glycol fusion (2) of CV-1 cells (permissive for SV40) to the SV40 but not the JCV transformants. A similar experiment using primary human fetal glial cells (permissive for JCV) was not successful due to the toxic effect of polyethylene glycol on these cells. Therefore, we do not know whether virus is rescuable from JCV transformants by this procedure.

Tumor antigens were immunoprecipitated from extracts of the JCV- and SV40-transformed cells with sera from hamsters bearing tumors induced by these viruses. Two proteins of 96,000 and 92,000 daltons were detected in all JCV transformants. In addition, some of these lines contained larger immunoprecipitable proteins (Fig. 1). Several proteins were also isolated from the SV40 transformants which were equal to or larger in size (108,000, 99,000, and 94,000 daltons) than the T antigen extracted from SV40-infected CV-1 cells (94,000 daltons, Fig. 1). One explanation for this variety of proteins is that more than one species of mRNA coding for T antigen is present in the transformed cells. This was previously shown to be the case in SV40-transformed mouse and rat cells in which several large T proteins were found (10, 19).

A small t protein was not detected in JCV transformants using JCV anti-T sera or an antiserum which recognizes SV40 small t (SV40 serum no. 1, kindly supplied by Kathleen Rundell). The small t protein of SV40 was observed in both lytically infected (Fig. 1) and trans-

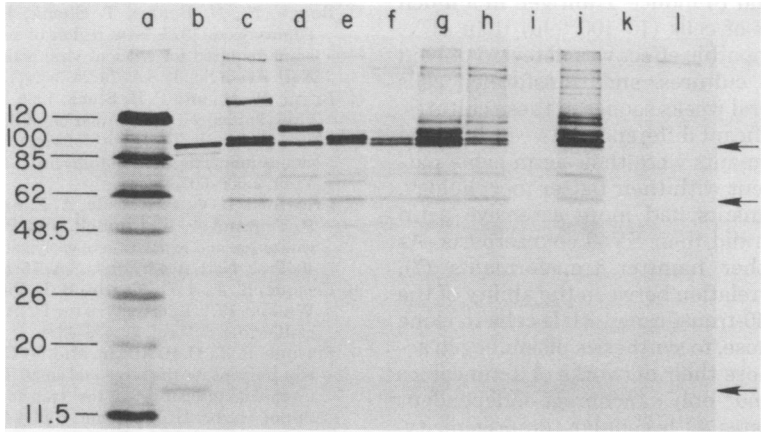

FIG. 1. *Immunoprecipitation of T antigens from PHB cells transformed by JCV and SV40. [^{35}S]methionine-labeled proteins from 5 × 10^5 normal or transformed cells were precipitated with anti-T serum as described by Lane and Robbins (12) and analyzed on 10 to 17.5% sodium dodecyl sulfate-polyacrylamide gels. Phenylmethylsulfonylfluoride (20 μg/ml), a protease inhibitor, was incorporated into the extraction buffer. (a) Proteins extracted from adenovirus type 2-infected HeLa cells 25 h postinfection served as molecular weight markers. The remaining tracts represent proteins precipitated from extracts of (b) SV40-infected CV-1 cells, (c) SV40-PHB cells, (d) C-SV40-PHB cells, (e) SV40 DNA-PHB cells, (f) HJC-15 tumor cells, (g) Mad 1-PHB cells, (h) C-Mad 1-PHB cells, (i) Mad 1 DNA-PHB cells, (j) Mad 2-PHB cells, (k) Mad 3-PHB cells, and (l) uninfected hamster embryo fibroblasts. Cellular extracts were precipitated with SV40 antiserum no. 1 (b), no. 2 (c to e), or JCV antiserum (f to l). Arrows refer to the positions of the large, middle, and small t proteins.*

formed (data not shown) cells using SV40 serum no. 1. Additional SV40 and JCV anti-T sera, however, failed to precipitate this protein from the SV40 transformants.

Our antisera also immunoprecipitated middle T (58,500 daltons; Fig. 1), a cellular protein induced by SV40 (11), and apparently also by JCV, in transformed cells. Middle T forms an oligomeric complex with large T and is therefore precipitated with anti-T sera (11). An antiserum which specifically recognizes cellular middle T also precipitates this protein from the JCV transformants (D. Lane, personal communication).

The variability in the quantity of the large and middle T proteins immunoprecipitated from different JCV transformants was reproducible. These proteins were detected in the Mad 1 DNA and Mad 3 transformants upon longer exposure of the gels (96 versus 16 h). The significant reduction of large and middle T in these two cell lines correlated with the observation that immunofluorescent staining of the T antigen in these cells (cloned or uncloned) revealed a heterogeneous fluorescence pattern. The vast majority of cells showed only a weak nuclear fluorescence; 1 to 5% of the cells stained with an intensity that was comparable to that of the other transformants.

This study reveals differences between the transformation of PHB cells by roughly equal titers of SV40 and JCV. Early after infection, SV40 was found to induce T antigen in a much larger number of cells (10–100 fold) than JCV. Extensive cytopathic effect was detected only in SV40-infected cultures, and transformed cells appeared several weeks sooner in these cultures. The only significant difference between JCV and SV40 transformants were their actin cable patterns. Consistent with their flatter morphology, JCV transformants had more extensive actin networks than did their SV40 counterparts. As shown for other hamster transformants (7), there is no correlation between the ability of the JCV- and SV40-transformed PHB cells to clone in methycellulose, to synthesize plasminogen activator, or to lose their networks of actin cables (Table 2), and only anchorage-independent growth correlates with cellular tumorigenicity. Both sets of transformants contain large and middle T proteins as detected by immunoprecipitation; however, small t has only been observed in the SV40-transformed cells.

Recently, variants of the human polyomavirus, BK virus, have been isolated (BKV MM; 23), and viable mutants of SV40 have been generated (dl 54/59; 21, 22), which have deleted sequences encoding their small t proteins (5, 22, 26). DNA extracted from the different isolates of JCV used in the present study is heterogeneous (6, 14, 15) and some populations of shorter molecules appear to be viable (6). If some of these variants of JCV are analogous to the small t variants of SV40 and BK virus, one might expect to observe some dependence on small t for the transformation of certain cells by JCV. Rat cells transformed by the dl 54/59 mutants of SV40 display a defective transformed phenotype (3, 22), whereas hamster cells transformed by these mutants are indistinguishable from their wild-type counterparts (7). Similarly, hamster cells transformed by JCV closely resemble SV40 transformants (Tables 1 and 2; Fig. 1). We are now attempting to transform rat cells with both JCV and SV40 to compare the phenotypes of such transformants.

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