Cell type-specific expression of JC virus T antigen in primary and established cell lines from transgenic mice

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The highly restricted host range of JC virus (JCV) has made it difficult to study the biology of this common human papovavirus. To increase our understanding of the tissue specificity of this virus, we have examined the expression of the T antigen (T-Ag) in primary and established cell lines from various tissues of transgenic mice containing the JCV early region. In contrast to earlier results from a simian virus 40-containing transgenic mouse, there was no T-Ag expression in mesenchymal fibroblasts derived from two lines of JCV-transgenic mice. Instead, we isolated T-Ag-positive (T-Ag$^+$) cells that had characteristics consistent with a neural crest origin. Furthermore, primary brain cultures contained many T-Ag$^+$ astrocytes, but no expression was detected in macrophages, epithelial cells, neuronal cells nor, surprisingly, in oligodendrocytes. Continued passage of these cultures resulted in vigorously growing glial fibrillary acidic protein-positive, T-Ag$^+$ astrocytes. Thus, the strict tissue specificity of JCV expression was maintained, despite the fact that the viral genome pre-existed in every tissue of these transgenic mice and these constraints on expression were preserved even when cells were explanted *in vitro*.

Introduction

JC virus (JCV) is a common human papovavirus that has significant antigenic and sequence homology with simian virus 40 (SV40) and BK virus (BKV) (Padgett & Walker, 1976; Tooze, 1981). Primary infections occur in over 70% of the population, apparently with no overt clinical symptoms, and JCV has been associated with the rare disease progressive multifocal leukoencephalopathy (PML), which occurs predominantly in severely immunocompromised patients. In PML, the virus can be isolated from demyelinated lesions within the white matter of the central nervous system (CNS). In these lesions, JCV replicates primarily in oligodendrocytes, the myelin-producing cells. Giant astrocytes with bizarre, hyperchromatic nuclei are often seen at the periphery of some lesions (Aksamit *et al.*, 1985; Richardson, 1961) and there is evidence that the JCV T antigen (T-Ag) is probably expressed in these cells (Mazlo & Tariska, 1982; Narayan, 1976; Stoner *et al.*, 1986). Intracerebral inoculation of JCV into newborn hamsters causes a diverse array of neural tumours (Padgett *et al.*, 1977*b*; Walker *et al.*, 1973), whereas intraperitoneal or subcutaneous inoculation results in a much lower rate of tumour induction and all of these tumours are sarcomas (Padgett & Walker, 1976). In primates JCV causes glioblastomas, after intracerebral inoculation into owl or squirrel monkeys (London *et al.*, 1978; Major *et al.*, 1987), but, although multiple glial tumours have been observed in patients with PML (Castaigne *et al.*, 1974; GiaRusso & Koeppen, 1978; Richardson, 1961), JCV has not been proven to be the cause of these or any other human tumours.

In contrast to SV40, JCV has a very limited host range *in vitro*, growing well only in primary human foetal glial cells (Frisque *et al.*, 1979; Padgett *et al.*, 1977*a*) and, although some replication has been demonstrated in several other cell types (Beckmann *et al.*, 1982; Fareed *et al.*, 1978; Takemoto *et al.*, 1979), JCV does not grow in the majority of cells tested (reviewed in Padgett & Walker, 1976). Transformation is also highly inefficient and restricted to a few cell types (Bollag *et al.*, 1989; Fareed *et al.*, 1978; Frisque *et al.*, 1980; Howley *et al.*, 1980; Mandl *et al.*, 1987; reviewed in Walker & Frisque, 1986).

In order to study differences in the tissue specificities and host ranges of the different papovaviruses, our laboratory has produced a series of transgenic mice

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containing the early regions of SV40, BKV and JCV (Small *et al.*, 1985, 1986*a*, *b*). SV40-containing transgenic mice, produced in our laboratory and others, characteristically developed choroid plexus papillomas (Brinster *et al.* 1984; Small *et al.*, 1985). Cell lines derived from a wide variety of tissues of an SV40-containing transgenic mouse rapidly acquired transformed properties upon serial passage *in vitro* (Small *et al.*, 1985). These tissues were histologically normal and T-Ag mRNA was not detectable in some of them, but explanted cells from brain, intestine, kidney, liver, muscle and spleen all expressed T-Ag within several passages of initiation. Although most of these cultures were probably fibroblasts, expression also occurred in other cell types and the non-adherent spleen cells had many characteristics of basophil/mast cells (Small *et al.*, 1985).

In contrast to the SV40-induced choroid plexus papillomas, mice containing the BKV early region developed primary renal tumours and hepatocellular carcinomas (Small *et al.*, 1986*a*). Fibroblasts from one of these mice, BK37, did not express T-Ag, although we have established lines of T-Ag-positive (T-Ag$^+$) epithelial cells from renal tumours of several BK37 mice (A. H. Beggs & G. A. Scangos, unpublished).

JCV-containing transgenic mice developed adrenal neuroblastomas that underwent metastasis to several other tissues (Small *et al.*, 1986*a*). In addition, two lines of mice (JC48 and JC91) both suffer from a dysmyelination syndrome, resulting in shaking and tonic seizures and leading to an early death (Small *et al.*, 1986*b*). Northern blot analysis demonstrated that the primary site of JCV T-Ag expression in these mice was the brain, whereas most other tissues expressed low or undetectable levels of T-Ag mRNA. In the brain, levels of the myelin-specific proteins, myelin basic protein (MBP) and proteolipid (PLP), were reduced, whereas the astrocyte-specific protein, glial fibrillary acidic protein (GFAP), was elevated in affected mice (J. A. Small *et al.*, unpublished). *In situ* hybridization to JCV T-Ag RNA in brain sections suggested that oligodendrocytes, the myelin-producing cells, expressed T-Ag, as expression was localized to white matter and myelinating tracts in a pattern similar to that seen for MBP and PLP messages (Trapp *et al.*, 1988). Furthermore, T-Ag mRNA was first detected at 6 days of age and levels peaked at 3 weeks, similar to the temporal patterns of MBP and PLP expression.

To study the tissue specificity of JCV T-Ag expression, we have examined primary and established cell cultures from various tissues of the transgenic mice that suffered from dysmyelination (Small *et al.*, 1986*b*). These mice contained only the early region of JCV, including the regulatory region at the origin of replication and the complete coding region for T-Ag. Thus, no genes for structural proteins were present and no productive infection could occur. In contrast to our previous results on the SV40-containing mouse M13, fibroblasts from two lines of JCV transgenic mice (JC91 and JC48) did not express T-Ag, even after extensive growth *in vitro*, but T-Ag$^+$ cells did grow out of muscle explants from JC91 mice. These transformed cells expressed mRNA for the nerve growth factor receptor, suggesting that they may be neural crest derivatives. Cytospins and primary cultures of dissociated brain cells from JC91 revealed that T-Ag was temporally regulated *in vivo* and that GFAP-positive astrocytes were capable of expressing T-Ag *in vitro*.

Methods

Cell cultures. Unless otherwise indicated, all cells were grown in Dulbecco's modified Eagle's (DME) medium supplemented with 10% foetal bovine serum (FBS), L-glutamine, penicillin (50 units/ml) and streptomycin (50 μg/ml) (all Gibco). Cultures were maintained at 37 °C under 5% CO_2 and 95% air. All cell lines were free from mycoplasma, as judged by the Hoechst 33258 fluorescence assay (Chen, 1977). HJC-15 cells, used as controls in some experiments, are derived from a JCV-induced hamster glioblastoma and have been extensively characterized elsewhere (Frisque *et al.*, 1980; Walker *et al.*, 1973).

All cell cultures were derived from hemizygous transgenic mice whose genotypes were confirmed by Southern blot analysis. Cultures from muscle explants were initiated by mincing a piece of abdominal muscle in growth medium and plating the tissue in T-80 flasks (Nunc). The cells were trypsinized and split after 7 to 10 days by counting in a haemocytometer and plating $5{\cdot}2 \times 10^3$ cells per cm^2 in T-25 or T-80 flasks. The 3T3 cultures were passaged in this manner every third day; the high density (HD) cultures were split 1:4 only after reaching confluence (every 3 days to 1 to 2 weeks, depending on the age of the culture). Serum-free medium was prepared as described (Beggs & Scangos, 1986).

Primary brain cultures were prepared by finely chopping the cerebral hemispheres of mice of indicated ages and digesting the tissue at 37 °C in collagenase (0·002%), trypsin (0·2%) and chicken serum (2·5%) (CTC) in calcium- and magnesium-free Hanks' buffered salt solution (HBSS) for 30 min. Brains from older mice were often digested with 2 washes of CTC for a total of 45 min. The tissue was centrifuged at 40 *g* for 10 min and washed in growth medium with serum supplemented with 40 μg/ml DNase (Sigma). The remaining clumps of tissue were broken up by gently triturating with a wide-bore pipette and the cell suspension was filtered through four or five layers of sterile cheese cloth. After two washes with growth medium, $0{\cdot}5 \times 10^5$ to 2×10^5 cells were plated on 22 mm glass coverslips coated with human fibronectin or poly(L-lysine) (both Collaborative Research). To induce development of oligodendrocytes, the cultures were grown in DME medium with 10% FBS supplemented with 5 mg/ml insulin (Collaborative Research), as described by McMorris *et al.* (1986).

Immunofluorescence. Cells on coverslips were washed with phosphate-buffered saline (PBS) and fixed with 50% methanol and 50% acetone for 90 s. After eight to 10 washes with PBS, the cells were incubated with primary antisera for 30 min washed two or three times with PBS and incubated with fluorescently labelled secondary antiserum for 15 min. Following several final rinses, the cells were viewed and photographed on a Leitz Flouvert epifluorescence microscope with Kodak Tri-X or Ektachrome 200 film. Anti-T-Ag

antiserum, a gift from G. Jay, was from an SV40-induced tumour-bearing hamster. This antiserum cross-reacts with SV40, JCV and BKV T-Ags. Anti-p53 mouse monoclonal antibody (MAb) was also a gift of G. Jay (Dippold *et al.*, 1981). Mouse MAbs against GFAP (clone G-A-5) and the medium M_r neurofilament (clone NN18), and goat anti-vimentin antiserum, were from ICN Biochemicals, rabbit anti-galactocerebroside was from Chemicon International and rabbit anti-cytokeratin was a gift from R. Eichner. Rabbit anti-human fibronectin was from Organon-Teknika and rhodamine-labelled phalloidin was a gift from B. Packard. Tetanus toxin receptors were visualized by incubating unfixed cultures with 10 μg/ml tetanus toxin (Calbiochem) for 30 min at 37 °C. After washing in HBSS, the cells were incubated with equine anti-tetanus toxin (Scalvo Biologicals) at 4 °C for 30 min, followed by three more washes and fixation as above. After fixation, the cells were stained for T-Ag and labelled with the appropriate secondary antiserum. Rhodamine- and fluorescein-labelled secondary antisera (Organon-Teknika) were used at dilutions of 1 : 100.

Immunoprecipitations. The immunoprecipitation protocol has been described (Loeb *et al.*, 1987). Briefly, exponentially growing cells were labelled for 4 h with 100 μCi/ml [^{35}S]methionine in methionine-free DME. Lysed cells were pre-adsorbed with Pansorbin (Calbiochem), incubated with either anti-T-Ag or anti-p53 antiserum and precipitated with Pansorbin. The precipitates were analysed by 10% SDS–PAGE, followed by enhancement with Amplify (Amersham) and exposure to preflashed Kodak XAR film at −70 °C in the presence of an intensifying screen.

DNA and RNA isolation and analysis. High M_r DNA from cell lines was isolated as described by Wigler *et al.* (1979). Restriction digests were carried out as recommended by the enzyme supplier (New England Biolabs) and 10 μg aliquots of DNA were separated on 1% agarose gels. Southern blots were performed as described by Wahl *et al.* (1979) and modified by Huttner *et al.* (1979). Nitrocellulose filters were hybridized for 16 h with nick-translated pJC2-7 (Howley *et al.*, 1980) in 2 × Denhardt's solution, 20 mM-sodium phosphate pH 6·5, 6 × SSC, 10% dextran sulphate and 100 μg/ml boiled salmon sperm DNA at 65 °C. The filters were washed three times in 2 × SSC, 0·1% SDS at room temperature and four times in 0·1 × SSC, 0·1% SDS at 65 °C, followed by autoradiography using an intensifying screen at −70 °C.

RNA was isolated by the method of Chirgwin *et al.* (1979). Total cellular RNA (15 μg) was adjusted to 50% formamide, 20 mM-MOPS pH 7·0, 5 mM-sodium acetate, 1 mM-EDTA and fractionated in a 1% agarose, 2·2 M-formaldehyde gel. The RNA was transferred to a nitrocellulose membrane and hybridized to ^{32}P-labelled pH3-1 (nerve growth factor receptor cDNA), as described by Johnson *et al.* (1986).

Results

Fibroblast cultures from JCV-containing transgenic mice do not express T-Ag

Offspring of transgenic founder mice contain the injected DNA in all of their cells and so provide a good system to study the basis for tissue-specific gene expression. Our previous experience with cell lines derived from an SV40-containing transgenic mouse suggested that many cell types were capable of expressing T-Ag *in vitro*, even though they did not express it *in vivo* (Small *et al.*, 1985). To determine the pattern of expression of the JCV T-Ag, which has a narrower host range *in vitro*, we established several primary fibroblast cultures from abdominal muscle explants of JC48 and JC91 mice, as well as from negative litter-mates. Cultures were initiated from two different animals of each genotype and, at the first passage, each culture was split and passaged following two protocols. Half of the cells (designated 3T3) were passaged quantitatively following the 3T3 transfer regimen of Todaro & Green (1963), which allowed the establishment of contact-inhibited fibroblasts without giving transformed cells a large growth advantage. The remainder of the cells were maintained at a high density by splitting them 1 : 4 only after they had been confluent for 1 or 2 days (designated HD). This protocol was designed to allow the overgrowth of transformed cells that had lost the density-dependent inhibition of growth. Thus, if a subpopulation of cells only expressed JCV T-Ag and a transformed phenotype, they should be selected for in these cultures.

All of the cell cultures entered crisis within the first few passages of initiation and both the HD and 3T3 cultures from JC48 mice were indistinguishable from the wild-type controls (wt). These cells were passaged for over 3 months and T-Ag$^+$ cells were never detected. Furthermore, the phenotype of these cells, with respect to transformation, was always similar to the control cultures (Table 1). A Southern blot probed for JCV sequences confirmed that all of the JC48 lines contained the JCV early region present in the transgenic animals (not shown).

Initially, the JC91 cultures were similar to the wt cells, but the JC91 HD cultures had developed two morphologically distinct subpopulations of cells by the fifth or sixth passage (Fig. 1). These consisted of small, refractile, spindle-shaped cells that grew on a background of flat, fibroblastic cells, and continued passage at high density enriched the small cells, as they were not contact-inhibited. When these cultures were stained for JCV T-Ag, the spindle-shaped cells were T-Ag$^+$, but the fibroblastic cells were not (Fig. 1). The JC91 3T3 cultures also contained some of these smaller cells at early passages, although by passage 25 one subculture of JC91-1 3T3 had lost this subpopulation and these cells remained a minority in the JC91-2 3T3 culture.

To determine whether these T-Ag$^+$ cells were initially present in these cultures, or whether they arose from T-Ag-negative (T-Ag$^-$) fibroblasts, we allowed late passage, T-Ag$^-$, JC91-1 3T3 cells to remain confluent. We were never able to rescue any T-Ag$^+$ cells from this culture, but in similar conditions rare T-Ag$^+$ cells in the JC91-2 3T3 cultures formed foci against a background of contact-inhibited fibroblasts. Thus, the fibroblasts were not spontaneously becoming transformed and T-Ag$^+$, suggesting that the T-Ag$^+$ cells represented a pre-existing subpopulation present in the original biopsy. When second passage JC91 cells were kept at confluence, only

Table 1. *Phenotypes of cell lines from muscle explants*

Cell line	Age of animal*	Passage	T-Ag-positive cells (%)	Growth in soft agar†	Tumorigenicity‡ (latency in days)
Wt-1 HD	15·5	24	<0·1	0·9	0/3
Wt-2 3T3	5	31	<0·1	<0·01	ND§
JC48-1 HD	3·5	31	<0·1	<0·01	ND
JC91-1 3T3	16	28	1·9	1·4	0/3
JC91-1 HD	16	18	61·1	5·7	3/3 (27)
JC91-2 HD	16	18	71·9	24·5	2/2 (61)
JC91-2 sc6	16	–	100	56·9	3/3 (57)
HJC-15‖	–	-	99·7	74·8	3/3 (13)

* Age (in weeks) of donor animal.

† Percentage of cells forming colonies of >50 cells scored at 3 weeks.

‡ Number of nude mice with tumours/number of animals injected (average time to detectable tumours). To confirm that tumours were caused by T-Ag⁺ cells, explants from JC91-2 HD and sc6-induced tumours were grown and stained for T-Ag. Both cultures contained many T-Ag⁺ cells.

§ ND, Not done.

‖ HJC-15 is a cell line derived from a JCV-induced hamster glial tumour (Walker *et al.*, 1973).

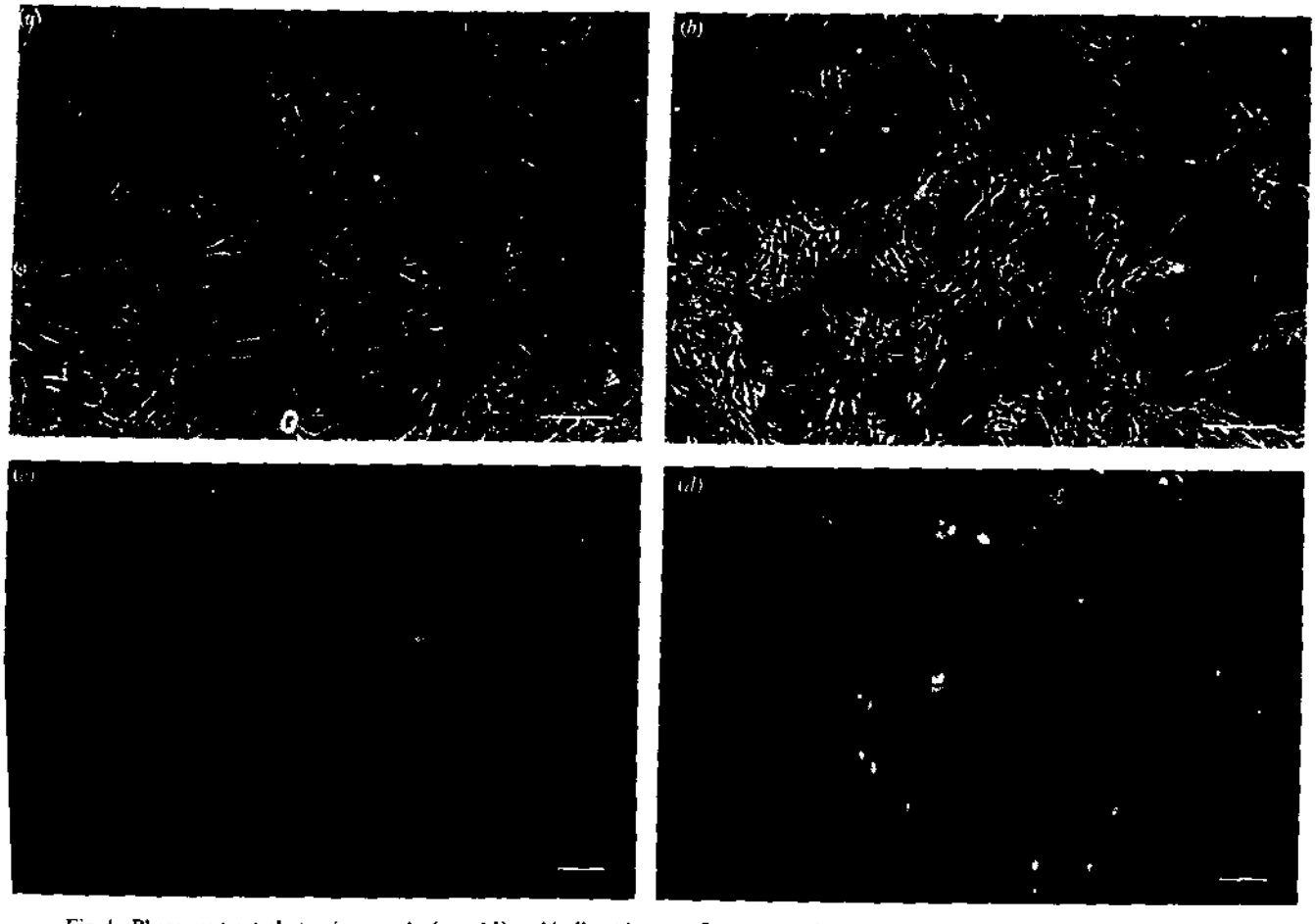


Fig. 1. Phase contrast photomicrographs (*a* and *b*) and indirect immunofluorescence for JCV T-Ag (*c* and *d*) of (*a*) JC91-1 3T3 p18, a pure culture of T-Ag⁻ fibroblasts, (*b*) JC91-1 HD P16 and (*c*) JC91-2 HD P10, both mixed cultures with refractile T-Ag⁺ cells growing on a monolayer of T-Ag⁻ fibroblasts; (*d*) JC91-1 sc6, a T-Ag⁺ subclone of JC91-1. Bar markers represent (*a*, *b*) 50 μm and (*c*, *d*) 100 μm.

two of the $1{\cdot}3 \times 10^5$ cells plated formed foci, indicating that these cells were initially quite rare.

Characterization of T-Ag⁺ cells from muscle explants of JC91 mice

Subclones of T-Ag⁺ cells from the JC91-2 culture were isolated to allow better characterization of these cells. All of the subclones were morphologically similar, consisting of highly refractile, spindle-shaped cells. Expression of T-Ag was stable, as almost all of the cells were T-Ag⁺ (Fig. 1*d*) and remained so upon subsequent passaging. In mixed cultures these cells preferentially grew on top of the flat fibroblasts, but in pure culture they did not attach to the plastic very well. Pretreating the dishes with collagen or laminin did not have a significant effect, but attachment and growth was enhanced on dishes pretreated with 5 μg/ml human fibronectin. When plated on glass coverslips, JC91-2 sc6 cells had no detectable actin cables, but plating on fibronectin-coated coverslips caused the cells to spread out, restoring some degree of organization to the cytoskeleton (compare Fig. 2*e* with 2*f*). Indirect immunofluorescence staining for extracellular fibronectin showed that these cells secreted very low levels of this protein compared to T-Ag⁻ JC91 fibroblasts (Fig. 2*a* and *d*). JC91-2 sc6 cells grew well in medium containing low serum concentrations, but were unable to grow in serum-free medium even when supplemented with fibroblast growth factor, insulin, transferrin and epidermal growth factor (data not shown). As 3T3 cells grow well in this medium (Beggs & Scangos, 1986) this result suggests that the JC91-2 sc6

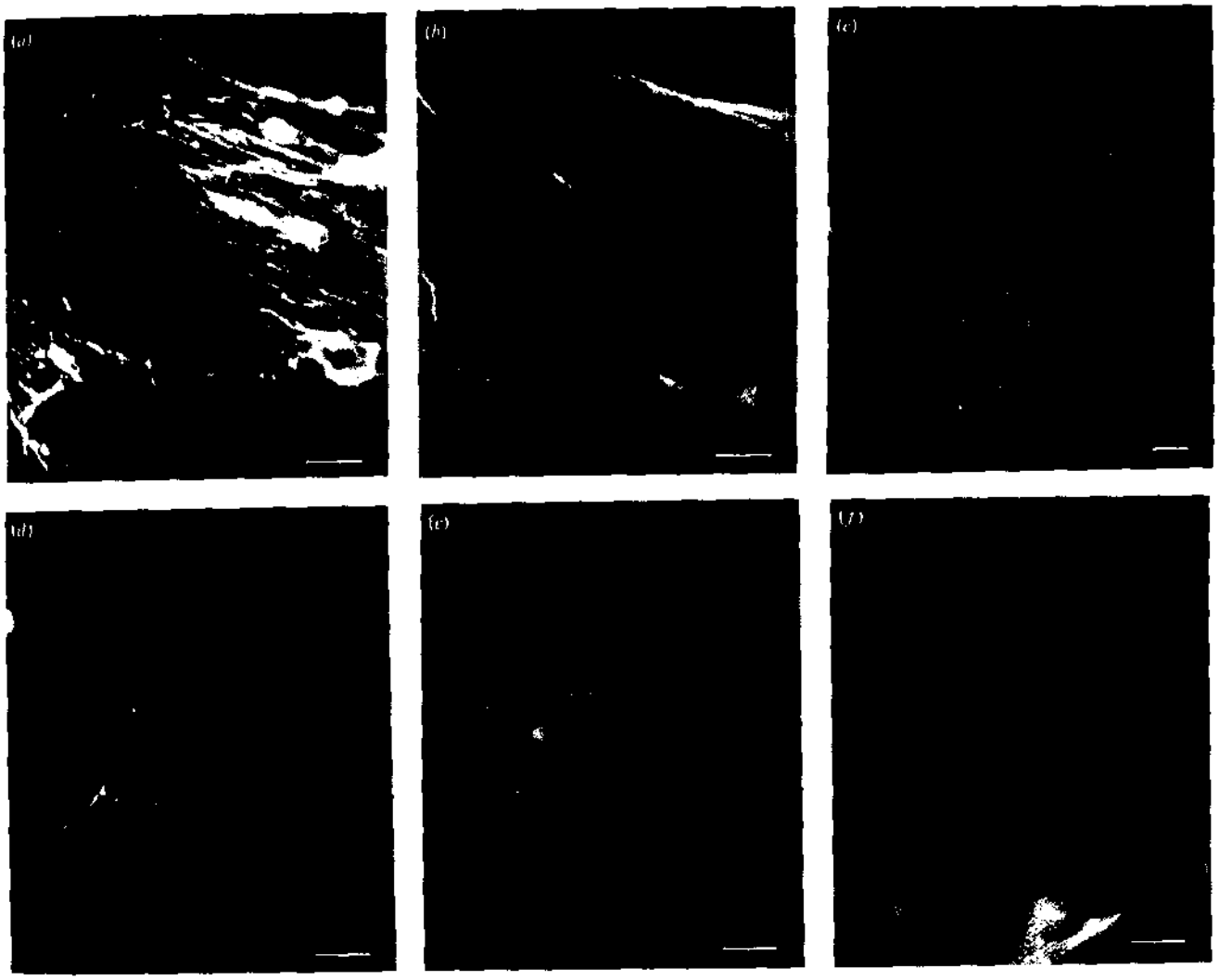


Fig. 2. Fluorescence photomicrographs of a T-Ag⁻ JC91-1 3T3 fibroblast stained for fibronectin (*a*), actin (*b*) and T-Ag-positive JC91-2 sc6 cells plated and stained for vimentin (*c*), fibronectin (*d*) and actin (*e*) and plated on a fibronectin-coated coverslip and stained for actin (*f*). Bar markers represent 50 μm.

cells require a serum growth or attachment factor that fibroblasts do not need.

These cells had many phenotypes common to papovavirus-transformed cells (Table 1). In addition to their growth characteristics and lack of cytoskeletal organization, they grew in soft agar suspension culture and formed fibrosarcomas when injected subcutaneously into nude mice.

To characterize further the T-Ag produced by JC91-2 sc6 cells, we immunoprecipitated ^{35}S-labelled protein extracts from these cells and several control cell lines. Analysis of extracts precipitated with anti-T-Ag antisera revealed that JCV T-Ag was present in both JC91-2 sc6 and HJC-15 cells and that it ran at a slightly lower apparent M_r than SV40 T-Ag (Fig. 3). No T-Ag was detected in JC91-1 3T3 cells, which contained less than 1% T-Ag$^+$ cells by immunofluorescence analysis. On our gels JCV T-Ag appeared to migrate as several bands of 90000 to 94000 M_r. In addition, a band corresponding to p53 was present in extracts precipitated with anti-T-Ag antiserum. As expected, this appeared slightly larger in HJC-15 cells, as hamster p53 is actually 55000 to 56000

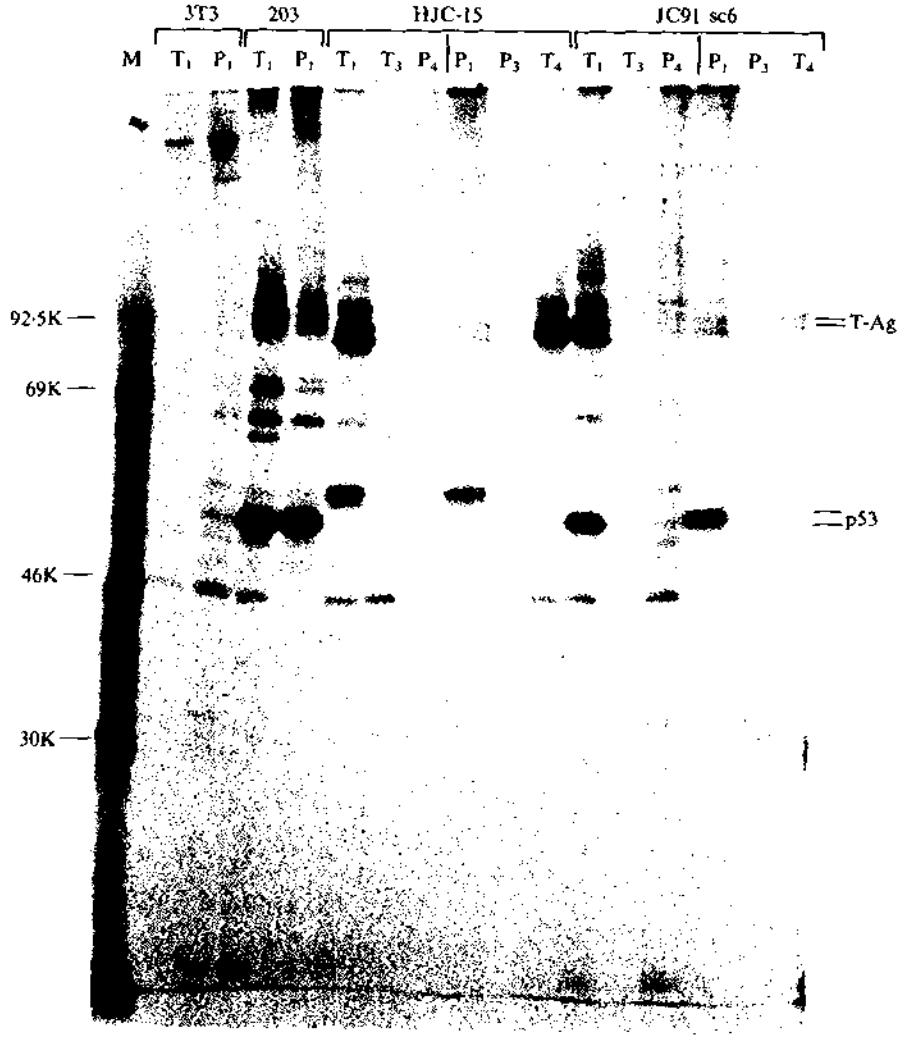


Fig. 3. Immunoprecipitation of T-Ag and p53. Cells used were: 3T3, JC91-1 3T3 P25 (<1% T-Ag$^+$ cells); 203, SV40-transformed BALB/c 3T3 cells; HJC-15, JCV-transformed hamster glioma cells; JC91-2 sc6 (100% T-Ag$^+$). ^{35}S-labelled cell lysates were immunoprecipitated with either hamster anti T-Ag antiserum (T) or mouse anti-p53 MAbs (P). HJC-15 and JC91-1 sc6 lysates were successively precipitated three times with anti-T-Ag, followed by anti-p53 [shown are 1st, 3rd and 4th precipitates (T_1, T_3 and P_4)]. The absence of precipitable p53 following exhaustive removal of T-Ag (P_4) demonstrates that all p53 was bound to T-Ag. Similar lysates were precipitated three times with anti-p53, followed by anti-T-Ag [shown are 1st, 3rd and 4th precipitates (P_1, P_3 and T_4)]. T-Ag in the final precipitation (T_4) demonstrates excess T-Ag unbound to p53. M, molecular size markers. The amount of T-Ag remaining after exhaustive precipitation of p53 in JC91 sc6 cells (last lane, T_4) is an underestimate due to sample loss; replicate experiments demonstrated that unbound T-Ag was present to the same extent as seen in HJC-15.

M_r (Jay *et al.*, 1981). To test whether these bands actually represented p53 we also precipitated extracts with a MAb against p53. This brought down not only the 53000 M_r band, but also some of the T-Ag, confirming that these two proteins were complexed. Exhaustive precipitation with first one antibody followed by the other revealed that T-Ag was present in excess in both the HJC-15 and the JC91-2 sc6 cells (Fig. 3).

A Southern blot of cell line DNAs, compared with tail DNA from a transgenic animal, revealed that the JCV sequences had undergone amplification and rearrangement in the cells that expressed T-Ag. Both HD cell lines contained new bands in *Pvu*II-digested DNA and the 2·5 and 0·6 kb fragments, derived from an intact early region, were darker than the corresponding fragments in tail DNA (Fig. 4*a*). The HD cell lines are mixed populations of T-Ag$^+$ and T-Ag$^-$ cells. DNAs from two T-Ag$^+$ subclones also exhibited these alterations, demonstrating that amplification was occurring in the T-Ag$^+$ cells. In contrast, the JC91-1 3T3 cell line which contained less than 1% T-Ag$^+$ cells, showed no evidence of amplification, although there was one new restriction fragment in *Bam*HI-digested DNA (not shown). When genomic DNAs from these cell lines were digested with an enzyme that does not cut within the transgene (*Msp*I), fragments of approximately 20 and 25 kb were detected and their intensity was increased in cells with amplified sequences (not shown). There was no evidence for free circular viral genomes in any cell lines, although one amplified cell line had a new *Msp*I fragment of 8 kb.

Because of the restricted tissue specificity of JCV, it was of interest to determine the origin of the T-Ag$^+$ cells that grew out of the muscle biopsies. Consistent with their mesenchymal derivation, indirect immunofluorescence for the intermediate filament vimentin stained JC91-2 sc6 cells brightly, although few organized fibres were detectable (Fig. 2*c*).

JCV is predominantly expressed in neural crest derivatives so we looked for expression of mRNA for the nerve growth factor (NGF) receptors, which are found on Schwann cells, melanocytes and other neural crest derivatives, but not on fibroblasts (Johnson *et al.*, 1986; Ross *et al.*, 1984). A band corresponding to 3·8 kb in RNA from JC91-2 sc6 and HJC-15 cells hybridized to a cDNA probe for the NGF receptor (Fig. 5). In contrast, no NGF receptor mRNA was detectable in several fibroblast lines, including T-Ag$^-$ cells from JC48 and JC91.

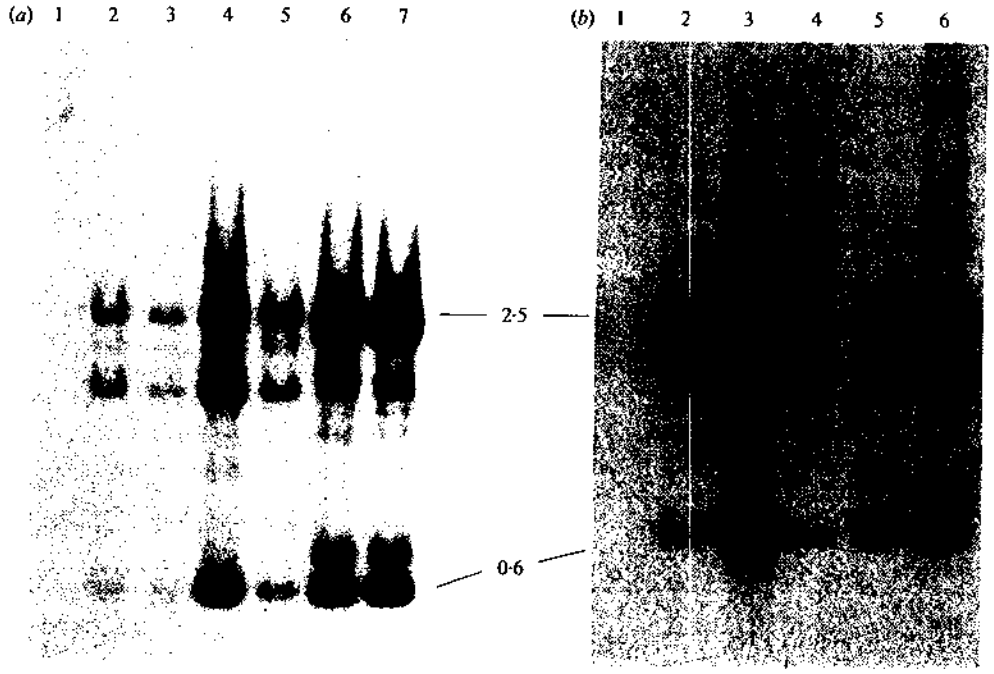


Fig. 4. Southern blot analysis for JCV sequences in T-Ag$^+$ and T-Ag$^-$ cell lines derived from muscle biopsies (*a*) and brain-derived cell lines (*b*). Total genomic DNA was digested with *Pvu*II. The 2.5 and 0.6 kb fragments derived from an intact early region of JCV are indicated. (*a*) Lanes: 1, wt-1 3T3 P17 (negative control); 2, JC91 mouse tail DNA; 3, JC91-1 3T3 P17, T-Ag$^-$ fibroblasts; 4, JC91 HD P14 and 5, JC91-2 HD P14, both mixed cultures with about 65% T-Ag$^+$ cells; 6, JC91-2 sc4 and 7, JC91-2 sc6, both T-Ag$^+$ subclones. The reduced intensity of bands in lane 3 relative to lane 2 reflects a lower amount of total DNA in that lane. All other lanes contained equal amounts of DNA. (*b*) Lanes: 1, wt-1 3T3 P17, negative control; 2, JC91 mouse tail DNA; 3, 91G9, GFAP$^+$, T-Ag$^+$ brain-derived cell line P10; 4, 91G10, similar to 91G9; 5, JC91-2 HD P14, about 65% T-Ag$^+$; 6, JC91-2 sc4, T-Ag$^+$ subclone.

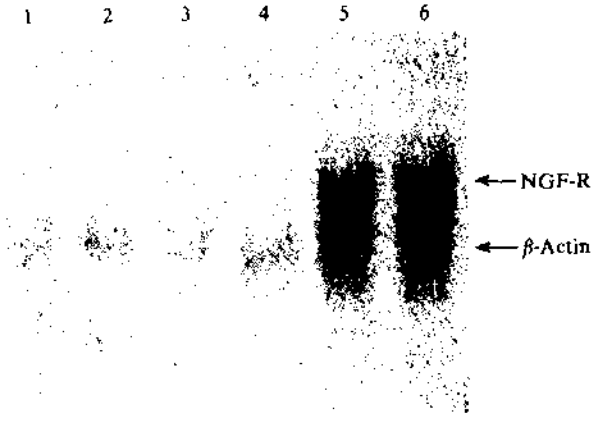


Fig. 5. Northern blot analysis for NGF receptor RNA in cell lines derived from muscle biopsies of wild-type and JCV-containing transgenic mice. Total cellular RNA was probed with a partial cDNA clone of the NGF receptor (pH3-1). Lanes 1 to 4 contain RNA from T-Ag⁻ cultures. Lanes: 1, wt-1 3T3 P31; 2, JC48-1 3T3 P17; 3, JC48-1 HD P17; 4, JC91-1 3T3 P31; 5, JC91-1 HD P14, a mixed culture with approximately 65% T-Ag⁺ cells; 6, HJC-15 hamster glioma. The 3·8 kb NGF receptor (NGF-R) and 2·1 kb β-actin transcripts (from a previous experiment) are indicated. The presence of β-actin mRNA controls for equal loading of each lane.

Expression of JCV T-Ag in primary and established brain cultures

Northern blot analysis has shown that the major site of T-Ag expression common to JC48 and JC91 mice is the brain (Small *et al.*, 1986*b*). To examine the temporal pattern of T-Ag expression *in vivo*, brains from JC91 mice of various ages were dissociated and cell suspensions were cytospun onto glass slides. Staining for T-Ag revealed positive cells only in mice 2 weeks old or older (Table 2). When cells from younger mice were cultured for several days, T-Ag⁺ cells appeared and the number of these cells was directly related to both the age of the animal and the length of time in culture. Thus, the developmental pattern of T-Ag expression *in vitro* paralleled that seen *in vivo*, albeit at a faster rate.

Table 2. *T-Ag expression in dissociated brain cells of JC91 mice*

Age of animals	T-Ag-positive cells (%)*		
	Cytospins	2 to 4 DIC†	6 to 8 DIC
1 day	<0·1	2·2 ± 1	9·5 ± 0·1‡
1 week	<0·1	8·2 ± 1‡	29·4 ± 7§
2 weeks	1·8 ± 0·5	12·1 ± 2	–
3 weeks	7·7 ± 0·4	–	–

* Five-hundred to 1000 cells per sample were scored. All values are means ± standard deviation of samples from three mice, unless otherwise indicated.

† DIC, Days in culture.

‡ Value is the mean of two samples.

§ Value is the mean of 12 samples, including data from Table 3.

To study the cell type specificity of T-Ag expression in these animals, we examined primary brain cultures from JC91 mice. These cultures were prepared from 1 week old mice and grown for 6 to 8 days *in vitro* prior to fixation. The cells were stained for T-Ag and various cell type-specific markers to determine which cells were capable of expressing T-Ag. These cultures contained a variety of different cell types and were typically composed of 30 to 40% T-Ag⁺ cells. As cell-specific markers, we used GFAP for astrocytes, galactocerebroside for oligodendrocytes and cytokeratin for epithelial cells (Osborn *et al.*, 1984; Raff *et al.*, 1979). We were also able to identify macrophages by their non-specific labelling with all of the secondary antibodies, giving a characteristic vacuolar staining pattern (Raff *et al.*, 1979). Up to 60% of the cells in these preparations were one of the above cell types and the rest were predominantly GFAP⁻ fibroblastic cells. Many of these secreted large strands of fibronectin and were probably fibroblasts (immunofluorescence data not shown), others may have been glial cell precursors. Staining with anti-neurofilament antisera revealed very few (if any) positive cells, indicating that neurons were generally not present in these cultures. This result was confirmed by staining for tetanus toxin receptors, which are found predominantly on neurons and also on some astrocytes and oligodendrocytes (Raff *et al.*, 1979, 1983). Tetanus toxin-positive cells in our cultures had the morphology of fibrous astrocytes or oligodendrocytes and not that of neurons (Fig. 7). The lack of neuronal cells was probably a result of the culture conditions (Prochiantz *et al.*, 1982) and has no bearing on the transgenic nature of the mice, as cultures from control mice were similar in this regard.

Double staining for GFAP and T-Ag revealed that 30 to 50% of GFAP⁺ astrocytes expressed T-Ag (Fig. 6 and Table 3) and these cells accounted for 60 to 88% of the T-Ag⁺ cells. Levels of expression were variable within groups of similar cells, probably because T-Ag was in the process of being turned on in these cells. Most of the T-Ag⁺ astrocytes were fibroblastic in morphology and probably corresponded to the protoplasmic astrocytes of grey matter (Raff *et al.*, 1983), but some process-bearing (fibrous) astrocytes were also T-Ag⁺. To confirm this, we stained these cells for tetanus toxin receptors, as these are found on protoplasmic, but not fibrous astrocytes (Raff *et al.*, 1983). Both tetanus toxin-positive and -negative cells expressed T-Ag, suggesting that both types of astrocytes were capable of expressing the JCV early region (Fig. 7).

The primary brain cultures were supplemented with 5 mg/ml insulin to enhance the growth of oligodendrocytes (McMorris *et al.*, 1986) and about 2% of the cells in

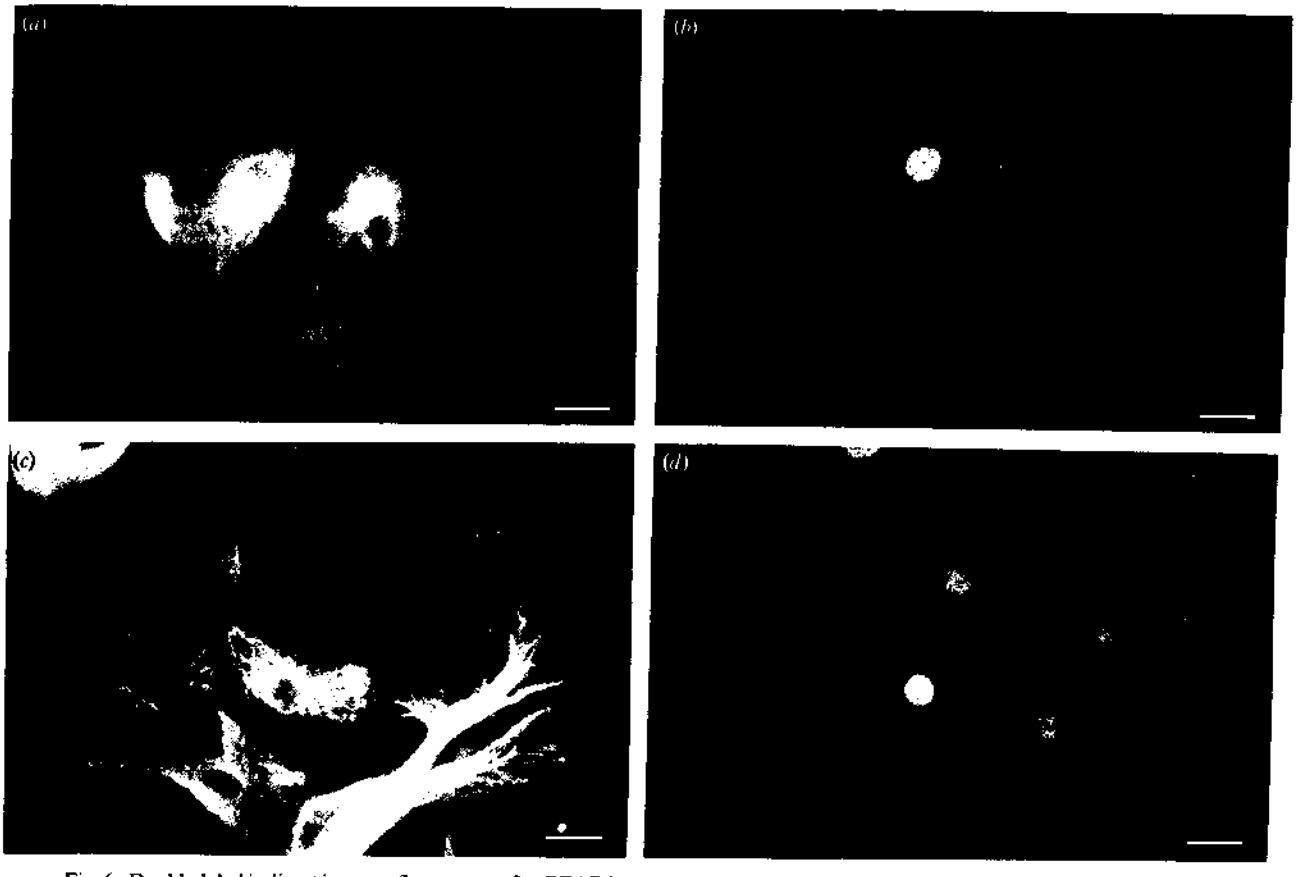


Fig. 6. Double-label indirect immunofluorescence for GFAP (*a* and *c*) and T-Ag (*b* and *d*) in primary brain cultures of JC91 mice. These panels illustrate the variability in expression of T-Ag among GFAP+ astrocytes. Cells were stained with mouse anti-GFAP, followed by fluoresceinated goat anti-mouse IgG and hamster anti-T-Ag followed by rhodamine-labelled goat anti-hamster IgG. Bar markers represent 50 μm.

Table 3. *Expression of T-Ag in JC91 brain cells* in vitro

Marker (cell type)	Cells with marker (%)*	T-Ag-positive cells (%)*	Marker-positive cells expressing T-Ag (%)*
GFAP (Astrocytes)	53 ± 13	27 ± 8	41 ± 11
Galactocerebroside (Oligodendrocytes)	2 ± 1	33 ± 4	<0·2
Cytokeratin (Epithelial cells)	2 ± 0·5	27 ± 8	<0·2
Vacuolar staining† (Macrophages)	2 ± 0·5	..	<0·2

* All values are means and standard deviations of counts on cultures from three or more animals. One-hundred to 1000 cells were scored for each sample.

† Data pooled from slides stained for galactocerebroside and cytokeratin. Four slides from two animals were scored.

these cultures were galactocerebroside-positive oligodendrocytes that grew on top of a monolayer of fibroblastic cells. These cells had large processes and their nuclei were often smaller than average. Double staining for galactocerebroside and T-Ag revealed that no oligodendrocytes contained detectable T-Ag (Fig. 8 and Table 3). This was surprising as JCV normally replicates in oligodendrocytes (Padgett & Walker, 1976) and T-Ag is apparently expressed *in vivo* in oligodendrocytes of JC91 and -48 (Trapp *et al.*, 1988). Nevertheless, no T-Ag+ galactocerebroside-positive cells were ever detected in any of our experiments.

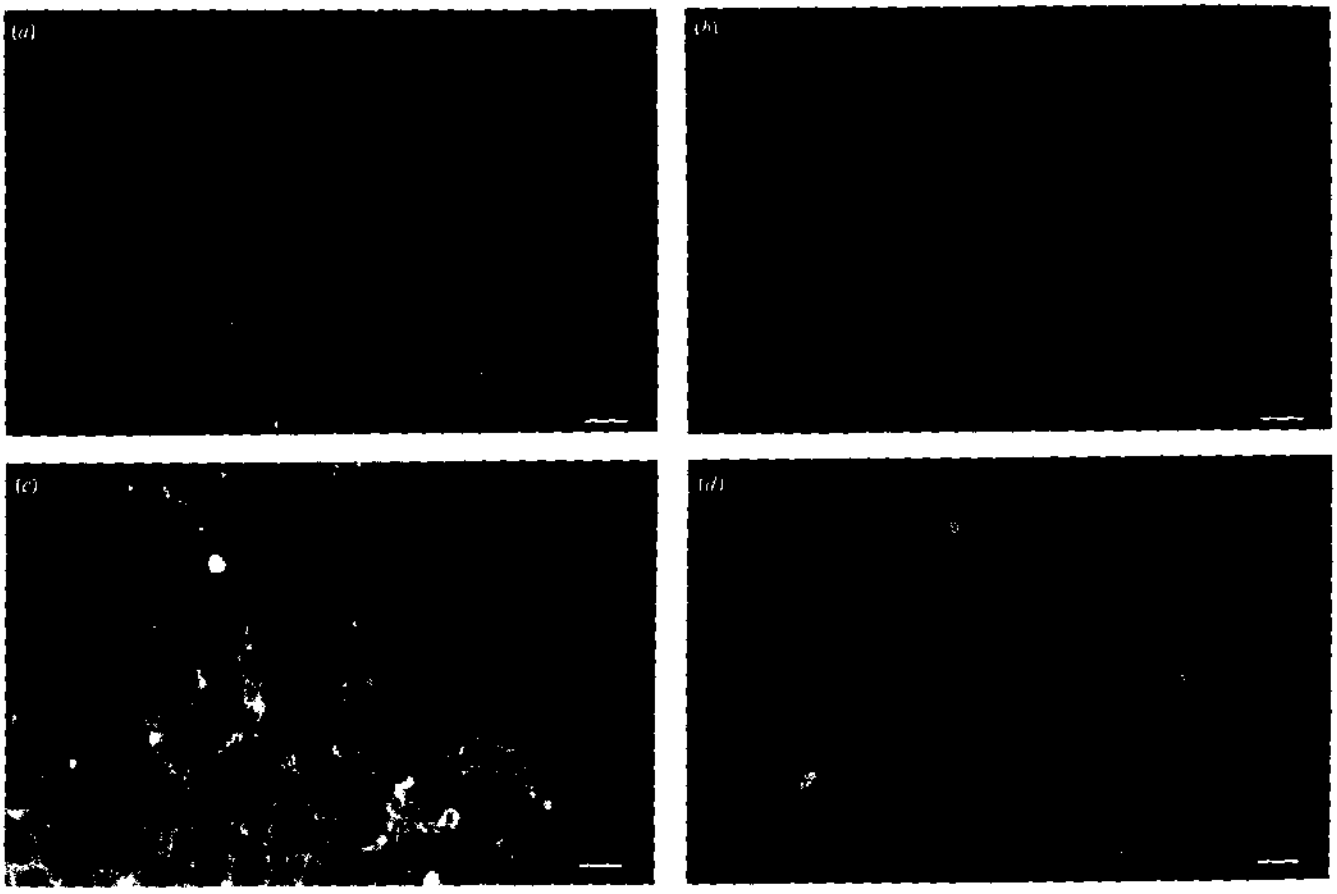


Fig. 7. Indirect immunofluorescence for tetanus toxin receptors (*a* and *c*) and T-Ag (*b* and *d*). (*a*) and (*b*) show a T-Ag$^-$, tetanus toxin receptor positive cell next to a group of T-Ag$^+$, tetanus toxin receptor-negative ones. (*c*) and (*d*) contain T-Ag$^+$, tetanus toxin receptor-positive cells. Cells were stained as described in Methods, using fluoresceinated goat anti-horse IgG to visualize tetanus toxin receptors and rhodamine-labelled goat anti-hamster IgG for T-Ag. Bar markers represent 50 μm.

Neither cytokeratin-positive, nor phagocytic, Fc receptor-bearing cells were stained for T-Ag, indicating that epithelial cells and macrophages did not express JCV T-Ag in these conditions (Table 3). Thus, up to 40% of the T-Ag$^+$ cells in these cultures were not identified with the markers we used. Double staining experiments with antiserum to fibronectin and T-Ag were difficult to interpret because some T-Ag$^+$ cells were growing in close association with fibronectin-secreting fibroblasts. Nevertheless, it seemed that T-Ag$^+$ cells did not secrete large strands of external fibronectin, suggesting that they were not fibroblasts.

Brain cultures from four JC91 and four control mice were split 1:4 at confluence and carried *in vitro* for several months. The control cultures all entered crisis after several passages and one of them did not survive. In contrast, all of the JC91 cultures contained a subpopulation of vigorously growing cells that did not go through crisis and the morphology of these cells ranged from flat and contact-inhibited, to round, refractile cells that grew in clumps. By the tenth passage, these lines contained 95 to 100% T-Ag$^+$ cells, all of which were GFAP$^+$. Staining for tetanus toxin receptors revealed that most cells were positive, although levels of staining were somewhat variable (data not shown). In contrast, the control cell lines were flat, contact-inhibited fibroblastic cells, similar in morphology to the *wt-1* 3T3 cells. None of these cells contained detectable GFAP, but large strands of extracellular fibronectin were present, consistent with these cells being fibroblasts.

A Southern blot of DNAs from two T-Ag$^+$, GFAP$^+$, brain-derived cell lines revealed that one of them contained new JCV-hybridizing bands, whereas the other had amplified and rearranged these sequences (Fig. 4*b*). Thus, it appears that instability of the transgenic JCV early region was a general property of JC91-derived, T-Ag$^+$ cell lines growing *in vitro*.

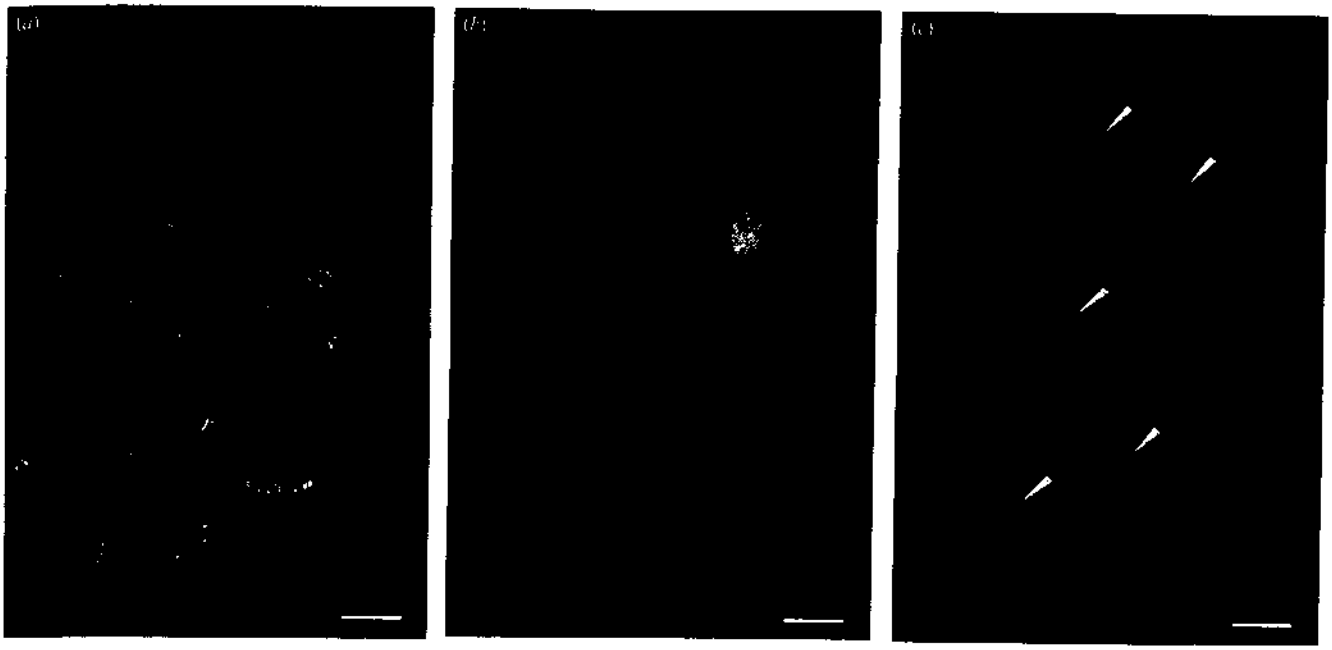


Fig. 8. Double label indirect immunofluorescence for galactocerebroside (*a*) and T-Ag (*b*) in primary brain cultures of JC91 mice. Panel (*c*) shows the same field stained with Hoechst 33258 to visualize nuclei. Arrows point to oligodendrocyte nuclei, which are small and slightly above the plane of focus. None of the oligodendrocyte nuclei express T-Ag. Rabbit anti-galactocerebroside was visualized using fluoresceinated goat anti-rabbit IgG. Bar markers represent 50 μm.

Discussion

We have examined expression of JCV T-Ag in primary cell cultures and established cell lines from various tissues of transgenic mice containing the JCV early region. In contrast to previous results on an SV40-containing transgenic mouse, expression of JCV T-Ag was not detected in early passage fibroblast cultures. However, a subpopulation of T-Ag$^+$ cells did grow out of some muscle cultures from JC91 mice. Similar cells were not detected in cultures from JC48 mice, which may reflect actual differences between the two lines of mice, perhaps related to the arrangement or site of integration of the transgenic JCV sequences. Alternatively, it may be related to the developmental stage of the explanted tissues as the JC48 mice were 3·5 weeks old, whereas the JC91 mice were 16 weeks old when these cultures were initiated. Unfortunately, offspring of the JC48 founder mouse did not live long enough to mate, so once JC48 died we were unable to repeat these cultures or test other tissues for expression *in vitro*.

Previous reports have demonstrated that JCV-transformed cells exhibit many properties characteristic of transformed cells *in vitro* (Frisque *et al.*, 1980; Major *et al.*, 1984, 1986). Our T-Ag$^+$ JC91 cells were not contact-inhibited, they contained a disorganized cytoskeleton (both actin and vimentin), they had a reduced serum requirement, they grew in soft agar and were tumorigenic in nude mice. However, as we do not have a non-transformed parental cell line available for comparison, we can not be sure that all of these phenotypes are a direct consequence of T-Ag expression, but this is likely as none of these properties were seen in any of the T-Ag$^-$ control cultures.

Unfortunately, the transformed nature of the JC91-2 sc6 cells has made it difficult to determine their cell type. Nevertheless, several lines of evidence support the idea that the T-Ag$^+$ cells in JC91 cultures were not derived from fibroblasts. The proportion of cells capable of expressing T-Ag was very low in early passage cultures, suggesting that these cells were a minor component of the original explant. If these cells were simply rare fibroblasts that had undergone some secondary change(s) allowing expression, then we should have been able to select out T-Ag$^+$ cells from pure cultures of JC-91 T-Ag$^-$ cells. Because we were unable to do this, and expression in T-Ag$^+$ subclones was stable, we believe that the T-Ag$^+$ cells were derived from a pre-existing subpopulation present in the original explants.

Expression of NGF receptors is normally limited to neuronal cells and some non-neuronal neural crest derivatives, including melanocytes and melanoma cells, pheochromocytoma cells, Schwann cells and neurofibroma cells (Ross *et al.*, 1984). Fibroblasts do not express NGF receptors or NGF mRNA, so the presence of NGF mRNA in JC91-2 sc6 cells provides strong evidence that

these cells are neural crest derivatives. This is an attractive hypothesis because JCV is normally expressed in neural crest derivatives. Furthermore, in our laboratory, four out of five transgenic mice containing JCV developed adrenal neuroblastomas (Small *et al.*,1986). Thus, although we can not rule out the possibility that expression of JCV T-Ag has induced NGF receptor expression, we feel that the T-Ag$^+$ cells in our cultures are probably derived from some non-fibroblastic cell type that expressed NGF receptors. By analogy to patterns of JCV T-Ag expression in the CNS, it is tempting to speculate that these cells may be related to Schwann cells or Schwann cell precursors, as these might have been present at low frequency in our initial explants.

The lack of JCV T-Ag expression in fibroblasts may be explained by observations we have made concerning a JCV T-Ag extinguisher function in this cell type. When we fused fibroblasts with JCV T-Ag$^+$ glial cells, expression of T-Ag was turned off in both heterokaryons and stable hybrids (Beggs *et al.*, 1988). This negative regulation helps explain the limited tissue-specificity of JCV and supports our view that JC91-2 sc6 cells probably represent a transformed derivative of some differentiated cell type other than fibroblasts.

Detection of elevated levels of p53 and its association with T-Ag in both JC91-2 sc6 and HJC cells was an interesting observation, since some previous reports have been unable to detect p53 in JCV-induced owl monkey glioma cells and in human foetal brain and kidney cells infected with JCV (Major *et al.*, 1984; Major & Traub, 1986). More recently, p53–T-Ag complexes were detected in JCV-transformed Rat 2 cells (Bollag *et al.*, 1989; Haggerty *et al.*, 1989). In this case, as with our HJC-15 and JC91 cells, the majority of T-Ag was not bound to p53, but it may be that differential binding of JCV T-Ag to p53 reflects species differences in p53, for example rodent p53 forms complexes with JCV T-Ag, whereas primate p53 does not.

Amplification and/or rearrangement of the transgenic JCV sequences occurred in all of the T-Ag$^+$ cell lines that we examined. Furthermore, preliminary data suggest that this may also occur in brains of older JC91 mice (E. H. Lee *et al.*, unpublished data). Similar alterations were previously seen in DNAs from adrenal neuroblastomas in two other JCV-containing transgenic mice (Small *et al.*, 1986*a*), as well as in JCV-transformed glial cells in culture (Mandl *et al.*, 1987). In each case, the intensity of restriction fragments corresponding to an intact early region was increased and often several new fragments were present. As these changes have been seen in three independent lines of mice, they are not unique to a particular integration site. Most probably, expression of T-Ag confers a selective advantage to a cell, so any cells that increase their levels of expression by amplification or rearrangement eventually predominate in populations of proliferating cells such as tumours or cell cultures.

Primary brain cultures from JC91 mice contained a variety of different cell types, but expression of T-Ag was apparently limited to astrocytes. These data conflict with observations *in vivo* by *in situ* hybridization to cells in brain sections of JC48 and -91 mice (Trapp *et al.*, 1988). JCV T-Ag RNA was detected only in white matter and myelinating tracts in a pattern very similar to that for the oligodendrocyte-specific proteins, myelin basic protein and proteolipid protein. Although expression was not detected in astrocytes, it is possible that T-Ag may have been expressed in only a subset of these cells, or that expression may have been at levels too low to detect. Alternatively, astrocytes may express T-Ag only under appropriate conditions. Culturing *in vitro* induced proliferation exposing them to serum factors with which they normally would not have had contact. Conversely, growing astrocytes *in vitro* may remove some negative influence that prevented expression of T-Ag *in vivo*. Our finding is not unique to JC91 mice, as we have also grown T-Ag$^+$, GFAP$^+$ astrocytes from brains of another line of transgenic mice containing the JCV MAD-11 early region (J. H. Miner & A. H. Beggs, unpublished data). Furthermore, a preliminary report has described cultivation of T-Ag$^+$ astrocytes from the brain of a human with SV40-associated PML (Narayan, 1976).

The lack of T-Ag expression in oligodendrocytes is somewhat more puzzling, as these cells definitely do express this protein *in vivo*. Our primary brain cultures were from 1 week old mice and many of the cells were undergoing differentiation and development during the time that they were in culture. In general, development of CNS macroglial cells from embryonic brains has been shown to be similar *in vivo* and *in vitro* (Abney *et al.*, 1981; Williams *et al.*, 1985). It is possible that the oligodendrocytes we identified were not at the proper stage of development to allow expression of T-Ag. At later time points, these cultures were overgrown by T-Ag$^+$ astrocytes, so other T-Ag$^+$ cells might not have been detected.

Of the T-Ag$^+$ cells in our cultures 10 to 40% were not identified with any of the cell type-specific antisera we used. In general, these cells were fibroblastic in morphology but, in contrast to other T-Ag$^-$ cells, they did not express large strands of extracellular fibronectin, suggesting they were not fibroblasts. These cells may have been immature GFAP$^-$ astrocytes in the process of development, since later passages of these cultures contained only GFAP$^+$, T-Ag$^+$ cells.

These data demonstrate that tissue specificity of JCV T-Ag expression was maintained even when cells were cultured *in vitro*. The growth advantage of T-Ag$^+$ cells allowed the outgrowth and characterization of cell types that would normally be overgrown by fibroblasts. In the

future, selective immortalization by introduction of transforming genes under the control of cell type-specific promoters may prove to be a useful tool in studying specific cell types that would otherwise not grow *in vitro*.

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