Journal of Cellular Biochemistry 67:223–230 (1997)

# Role of Cell Cycle Regulators in Tumor Formation in Transgenic Mice Expressing the Human Neurotropic Virus, JCV, Early Protein

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**Abstract** Transgenic mice harboring the early genome from the human neurotropic JC virus, JCV, develop massive abdominal tumors of neural crest origin during 6–8 months after birth and succumb to death a few weeks later. The viral early protein, T-antigen, which possesses the ability to transform cells of neural origin, is highly expressed in the tumor cells. Immunoblot analysis of protein extract from tumor tissue shows high level expression of the tumor suppressor protein, p53, in complex with T-antigen. Expression of p21, a downstream target for p53, which controls cell cycle progression by regulating the activity of cyclins and their associated kinases during the G1 phase, is extremely low in the tumor cells. Whereas the level of expression and activity of cyclin D1 and its associated kinase, cdk6, was modest in tumor cells, both cyclin A and E, and their kinase partners, cdk2 and cdk4, were highly expressed and exhibited significant kinase activity. The retinoblastoma gene product, pRb, which upon phosphorylation by cyclins:cdk induces rapid cell proliferation, was found in the phosphorylated state in tumor cell extracts, and was detected in association with JCV T-antigen. The transcription factor, E2F-1, which dissociates from the pRb–E2F-1 complex and stimulates S phase-specific genes upon phosphorylation of pRb and/or complexation of pRb with the viral transforming protein, was highly expressed in tumor cells. Accordingly, high level expression of the E2F-1-responsive gene, proliferating cell nuclear antigen (PCNA), was detected in the tumor cells. These observations suggest a potential regulating pathway that, upon expression of JCV T-antigen, induces formation and progression of tumors of neural origin in a whole animal system. J Cell. Biochem. 67:223–230, 1997. 

**Key words:** human JC virus; p53; T-antigen; transgenic mice; tumor cells

Tumors of neural origin are among the most common malignancies seen in both children and adults. Analysis of the genetic abnormalities that underlie such tumors has implicated a functional role for several oncogenes and tumor suppressor genes, including p53 and pRb [for review, see Wong et al., 1994]. De-regulation of both p53 and pRb has been extensively examined, with indications of a high incidence of mutations in these genes in astrocytoma [Wong et al., 1994]. The paradigm of virus-induced tumors represents an excellent model system for in vivo analysis of tumor progression. The association of viral oncogenic proteins, including SV40 T antigen and adenovirus E1A, with p53 and pRb, which may functionally inactivate these cell proliferation controllers is reminiscent of de-regulatory events observed in human tumors [Lang et al., 1994; Manfredi et al., 1991; Schlegel et al., 1993]. While the association between viruses and human tumors of neural origin has been weakly established, there have been several documented cases of patients with central nervous system (CNS) neoplasms

Contract grant sponsor: National Institutes of Health.

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Received 6 June 1997; accepted 26 June 1997

and reactivation of papovaviruses including simian virus 40 (SV40) and JC virus (JCV) [Bergsagel et al., 1992; GiaRusso et al., 1978; Lednicky et al., 1995; Rencic et al., 1996; Sima et al., 1983].

JCV is a human papovavirus known to cause the fatal demyelinating disease, progressive multifocal leukoencephalopathy (PML) [Padgett et al., 1971; ZuRhein and Chou, 1965]. Similar to other papovaviruses, the early region of the viral genome encoding the early protein, T-antigen is expressed prior to viral DNA replication. The early genome is separated by the viral regulatory sequences from the late region, which is responsible for production of the capsid proteins during the late phase of the viral lytic cycle [for review, see Frisque and White, 1992]. The neuro-oncogenic potential of JCV has been well established in several experimental animals, including Syrian hamsters, owl, and squirrel monkeys [London et al., 1978; Miller et al., 1984; Nagashima et al., 1984; Varakis et al., 1976; Walker et al., 1973; ZuRhein and Varakis, 1975, 1979]. The ability of JCV to induce neural origin tumors is likely attributed to the viral early protein, T-antigen, whose expression is restricted to neural cells [Raj and Khalili, 1995] and exhibits greater that 75% sequence homology to its well-characterized SV40 counterpart [Frisque et al., 1984]. In support of this concept, earlier reports along with our recent findings [Franks et al., 1996; Small et al., 1986] indicate that expression of JCV T-antigen in mice leads to the development of neural crest origin tumors in which the JCV early protein is highly expressed in the tumor tissue. This paper presents the results of our biochemical analysis of tumor tissue from transgenic mice in which levels of expression and activity of several cell cycle regulatory proteins including the tumor suppressors p53 and pRb have been evaluated.

## METHODS

### Transgenic Mice

Transgenic mice were generated by conventional methods as described previously [Gordon and Ruddle, 1983]. Briefly, a 3.2-kb *Bal*I/*Nci*I DNA restriction fragment of the JCV early-control region and the coding sequence for the viral early gene was injected into fertilized mouse oocytes generated by FVB/N mouse mating. The transgenic animals were identified by Southern blot analysis of DNA isolated from the tails upon treatment with *Eco*RI. The positive mice were observed daily for phenotypic manifestations, i.e., sluggish appearance with disheveled fur and distended lower abdomen, and formation of tumors as described previously [Franks et al., 1996].

### Reagents

Monoclonal antibodies to SV40 T-antigen (Ab-2) and to p53 (Ab-1) were obtained from Oncogene Science. Purified mouse anti-human Rb (G3-245) detecting mouse Rb protein was obtained from Pharminigen (San Diego, CA). Antibodies to cyclins, cyclin-dependent kinases (cdks), E2F (KH95), PCNA (PC10), and p21 (M-164) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

### Protein Extract Preparation and Analysis

Total protein extracts were prepared from tumor, brain, spleen, and kidney of adult transgenic mice and age-matched control animals by the method described previously [Franks et al., 1996]. Briefly, 0.5 mg of tissue was homogenized in TNN buffer containing 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.05% NP40, and a mixture of protease inhibitor containing aprotinin 2 μg/ml, leupeptin 10 μg/ml, pepstatin 10 μg/ml, PMSF 100 μg/ml, and TPCK 100 μg/ml. The homogenate was centrifuged for 15 min at 14,000 rpm, and the supernatant was collected and stored at −70°C for further analysis. All procedures for protein preparation were performed at 4°C. To measure the level of protein by direct Western blot analysis, approximately 50 μg of protein was fractionated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and, after transfer to nitrocellulose, reacted with the specific antibodies as described previously [Franks et al., 1996]. To examine the association of T-antigen with p53 or pRb, 200 μg of nuclear extract were incubated with anti-T-antigen, anti-p53, or anti-pRb antibodies overnight and the immune complexes pulled down with Pansorbin and washed and analyzed by immunoblotting with the secondary monospecific antibodies. Proteins were detected by enhanced chemi luminescence (ECL, Amersham, Arlington Heights, IL).

### Assay of Kinase Activity

Kinase activity was measured as a result of histone H1 phosphorylation in the presence of a given kinase or cdk as described previously [Kim et al., 1994]. Briefly, 150 μg of total pro-

tein extract from normal and transgenic tissues was immunoprecipitated overnight at 4°C with specific antibodies. Twenty µl of a 50% suspension of packed protein A–Sepharose beads in TNN lysis buffer was added and incubated at 4°C for 1 h. The immunoprecipitates were assayed for kinase activity for 20 min in reaction buffer containing 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 50 µM ATP, 5 µCi [$\gamma^{32}$P]-ATP, and 200 µg/ml calf thymus histone H1 (Sigma, St. Louis, MO) in a final volume of 50 µl. The reaction was carried out at 30°C for 20 min and stopped by the addition of 50 µl of sample loading buffer. Phosphorylated histone H1 was identified following SDS–PAGE and autoradiography.

## RESULTS AND DISCUSSION

The large T-antigen of JCV is composed of 695 amino acids with 70% sequence homology to the well-characterized SV40 large T-antigen [Frisque et al., 1984]. One of the most highly homologous regions between JCV and SV40 T-antigen (amino acid residues 259–517) partially overlaps with the region important for complex formation with the cellular tumor suppressor protein, p53. Despite sequence conservation in the regions encompassing p53 binding sites, the interaction between p53 and JCV T-antigen has not been completely established. In earlier studies, analysis of protein from owl monkey brain tumors induced by intracerebral inoculation of JCV revealed nuclear expression of JCV T-antigen, which was not associated with the host p53 protein [Major and Traub, 1986; Major et al., 1984]. However, in later studies, the association of JCV T-antigen with p53 was detected in cell extracts from a glioblastoma developed upon intracerebral inoculation of a juvenile owl monkey with a cell suspension of an explanted JCV-induced glioma [Major et al., 1987]. In order to determine the level of p53 in tumors developed in the JCV T-antigen transgenic mice and to assess its association with JCV T-antigen, protein extract from tumor and several nontumor tissues were prepared and reacted with anti-p53 and anti-T-antigen antibodies and immunocomplexes were analyzed by Western blot utilizing antibodies against T-antigen and p53, respectively. Figure 1A illustrates results from immunoprecipitation, using anti-p53 antibody (lanes 1–4) and anti-T-antigen antibody (lane 5), followed by Western blot using anti-p53 antibody. As evident from this study, in contrast to its undetectable level in brain, spleen, and kidney, substantial levels of p53 are expressed in tumor tissue (compare lanes 1–3 with lane 4) and significant amounts of this protein were found associated with T-antigen (compare lanes 4 and 5). It is likely that the association of T-antigen with p53 prolongs the half-life of p53 and results in the high-level accumulation of this protein in tumor tissue. In the reciprocal experiment, the level of T-antigen and its association with p53 was determined by immunoprecipitation and Western blot. As shown in Figure 1B, an extremely low level of T-antigen was detected in brain and kidney, but not in the spleen of transgenic mice (lanes 1–3). JCV T-antigen was highly expressed in tumor tissue and was found in complex with p53 (Fig. 1B, lanes 4 and 5).

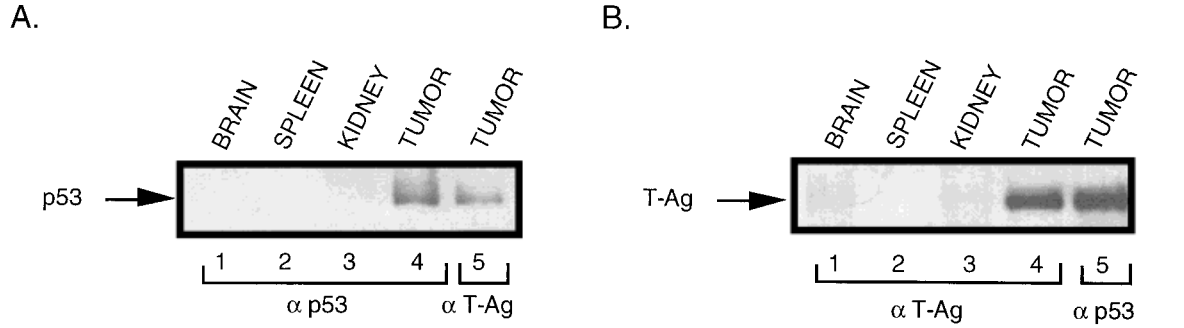


**Fig. 1.** Expression of p53 and JCV T-antigen in tumor and nontumor tissue of a transgenic animal. **A:** Protein extracts from brain, spleen, kidney and tumor of JCV T-antigen transgenic mice were immunoprecipitated with monoclonal antibody against p53 (**lanes 1–4**) or with monoclonal antibody against SV40 T-antigen that cross reacts with JCV T-antigen (**lane 5**). Immunocomplexes were resolved by SDS–PAGE and, after transfer to nitrocellulose, were subjected to Western blot analysis with antibody detecting p53. **B:** Protein extracts from brain, spleen, kidney, and tumor of transgenic mice were incubated with monoclonal antibody against SV40 T-antigen (**lanes 1–4**) or with monoclonal antibody against p53 (**lane 5**), and immunoprecipitates were subjected to Western blot analysis using anti-T-antigen antibody.

Previous studies have indicated that expression of p21, an inhibitor of cell proliferation, is upregulated by p53 [for review, see Cox and Lane, 1995; Hartwell and Kastan, 1994]. To examine the level of p21 in tumor tissue where a high amount of p53 has been detected in association with T-antigen, we performed Western blot analysis of protein extracts from tumor and nontumor tissues obtained from transgenic and age-matched control animals. As shown in Figure 2, despite a high level of p53 in the tumor tissue, extremely low levels of p21 were detected in these cells, suggesting that upon its association with JCV T-antigen, p53 may lose its ability to stimulate transcription of the p21 gene. Also, we observed that the level of p21 is significantly higher in the brains of transgenic mice compared to its level in age-matched control mouse brain. This is an intriguing observation, suggesting that overexpression of p21, through a p53 independent pathway, may participate in a pathway that prevents formation of tumors in the brains of transgenic mice.

p21 prevents cell cycle progression by binding to regulatory complexes composed of one of the cyclins and their partner kinases, cyclin dependent kinases (cdks) [Scherr, 1996]. Therefore, in the next series of studies, we assessed the level and activity of cyclins and their associated kinases in tissue from transgenic and age-matched control animals. As shown in Figure 3A, a high level of cyclin E was detected in tumor tissues. No dramatic variations in the level of cyclin E were observed in various tissues from transgenic mice versus those from control animals. The examination of H1 kinase activity of complexes obtained by immunoprecipitation with anti-cyclin E antibody revealed substantial kinase activity in extracts from tumor tissue as well as in brains and spleens of transgenic mice. Of interest, in control mouse brain where the level of cyclin E was comparable to that from transgenic animals, cyclin E exhibited no kinase activity. The evaluation of cyclin A expression by Western blot and its kinase activity by H1 kinase assay revealed a pattern very similar to that described for cyclin E. As shown in Figure 3B, a high level of cyclin A which correlated with its kinase activity was observed in the tumor tissue (lane 7). Similar to cyclin E, significant kinase activity was associated with the cyclin A complex obtained from the brains of transgenic mice. Next, we evaluated the kinase activity of cdk2, a cyclin-dependent kinase which is found in complex with cyclins E and A during G1 and S phases, respectively. As illustrated in Figure 3C, different levels of cdk2 were detected in various tissues from transgenic mice, and all were comparable to those seen in tissues from control animals. Of interest, the cdk2 complex from tumor tissue exhibited high levels of kinase activity as determined by H1 kinase assay (lane 7). These observations suggest that in non-tumor tissue cdk2 may be associated with inhibitors which prevent its kinase ability.

We next extended our studies and focused our attention on the early G1 regulators, including cyclin D and its associated kinases, cdk4 and cdk6. As demonstrated in Figure 4A, cyclin D1 was expressed in a variety of tissues, including tumor and exhibited a modest kinase activity. Similarly, as shown in Figure 4B, cdk4 was produced in a variety of tissues from control and transgenic mice with the highest levels observed in tumor cells. Interestingly, in contrast to its high level, no significant kinase activity was associated with the cdk4 complexes in tumor and non-tumor tissue. Analysis of cdk6 expression indicates lower levels of cdk6 in tumor and in brains of normal and transgenic mice (Fig. 4C). Regardless of its high levels in spleen and kidney, cdk6 showed no significant kinase activity in these cells. The cdk6 complex from brain, spleen, and tumor of transgenic mouse exhibits a modest kinase activity (Fig. 4C).

In the next series of experiments, we raised the question of whether the observed increased kinase activity of cyclin E, cyclin A, and cdk2 in tumor tissue correlates with the levels of phosphorylated pRb, a tumor suppressor which upon

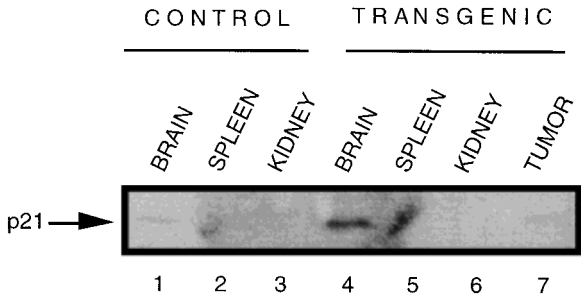


**Fig. 2.** Expression of p21 in various tissues from transgenic and control age-matched mice. Equal amounts of crude protein extracts (50 μg) from various tissues of normal (**lanes 1–3**) and transgenic animals (**lanes 4–7**) were analyzed by Western blotting using anti-p21 antibody.

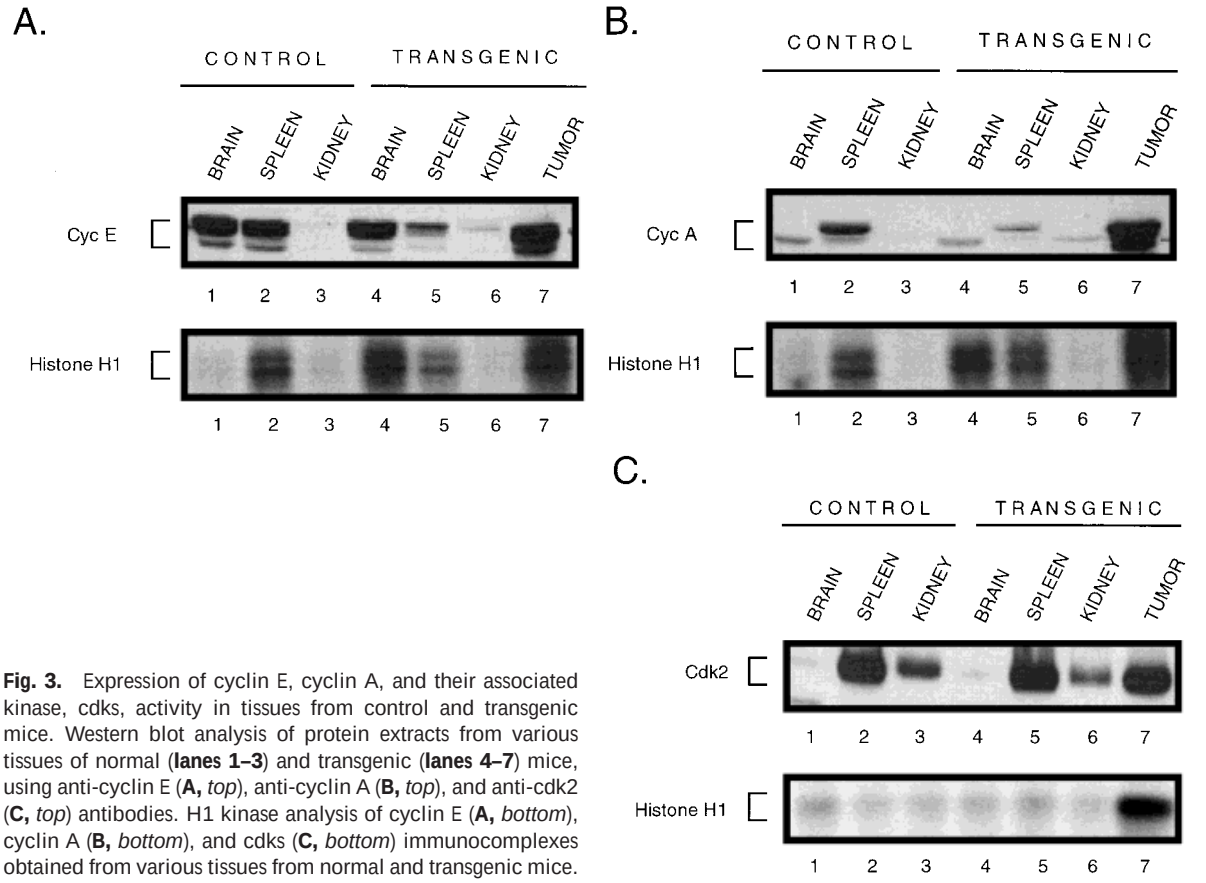


**Fig. 3.** Expression of cyclin E, cyclin A, and their associated kinase, cdks, activity in tissues from control and transgenic mice. Western blot analysis of protein extracts from various tissues of normal (**lanes 1–3**) and transgenic (**lanes 4–7**) mice, using anti-cyclin E (**A,** *top*), anti-cyclin A (**B,** *top*), and anti-cdk2 (**C,** *top*) antibodies. H1 kinase analysis of cyclin E (**A,** *bottom*), cyclin A (**B,** *bottom*), and cdks (**C,** *bottom*) immunocomplexes obtained from various tissues from normal and transgenic mice.

phosphorylation loses its ability to restrain cells in the G1 phase [Scherr, 1996]. Also, as complexation of several viral oncoproteins, including JCV T-antigen with pRb, may lead to functional inactivation of pRb, as well as induction of uncontrolled cell proliferation [Scherr, 1996], we sought to examine the state of pRb phosphorylation and the level of its association with JCV T-antigen. As shown in Figure 5, whereas a hypophosphorylated form of pRb was detected in a variety of tissues from transgenic mice, extracts from tumor tissue showed an additional band corresponding to the phosphorylated form of pRb. Moreover, results from Western blot analysis of the protein complexes pulled down by anti-T-antigen antibody suggest that JCV T-antigen may remain in complex with pRb in tumor tissue (Fig. 5A, lane 5). Results from the reciprocal experiment in which anti-T-antigen-specific immunocomplexes were analyzed for the presence of pRb verified the association of pRb and T-antigen in extract from tumor tissue.

One consequence of pRb phosphorylation and/or its association with JCV T-antigen would be liberation of E2F-1 from the pRb–E2F-1 complex. E2F-1 is a DNA binding transcription factor which upon binding to its consensus sequence stimulates expression of a group of S phase specific genes including its own. Hence, in the next study, we determined the level of E2F-1 in tissues from transgenic animals. As shown in Figure 6A, whereas the lowest level of E2F-1 was detected in the brain of the transgenic animal (lane 1), a high level of E2F-1 was observed in tumor tissue (lane 4). Thus it is likely that the liberated E2F-1 in tumor cells positively autoregulates transcription of its own promoter and results in high level production of this protein in tumor tissue.

In the last series of studies, we examined the level of proliferating cell nuclear antigen (PCNA), a protein whose expression is enhanced by E2F-1 during the S phase [Scherr, 1996]. As shown in Figure 6B, significant amounts of PCNA were detected in tumor tissue (lane 7). The level of PCNA in other tissues from transgenic mice was comparable to those from control animals.

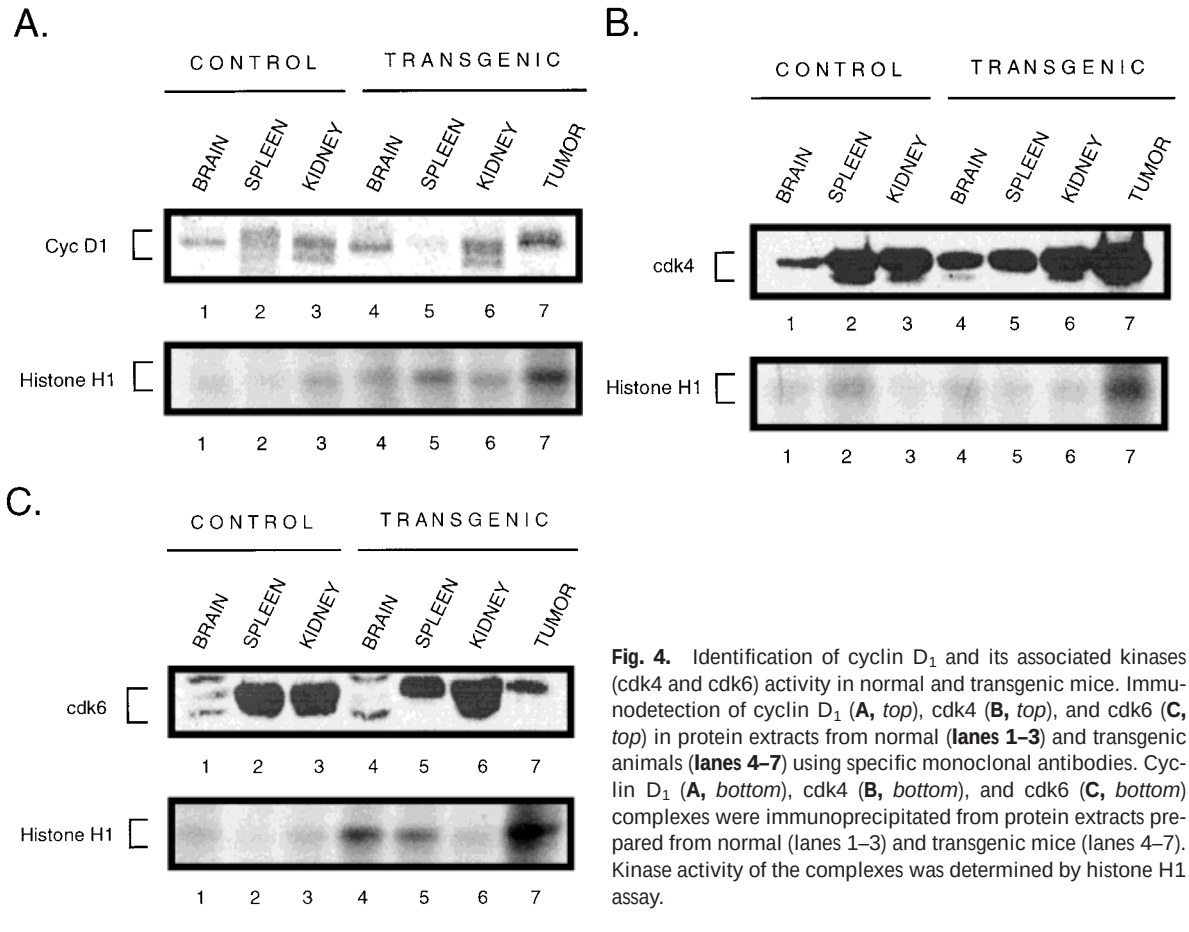


**Fig. 4.** Identification of cyclin $D_1$ and its associated kinases (cdk4 and cdk6) activity in normal and transgenic mice. Immunodetection of cyclin $D_1$ (**A,** *top*), cdk4 (**B,** *top*), and cdk6 (**C,** *top*) in protein extracts from normal (**lanes 1–3**) and transgenic animals (**lanes 4–7**) using specific monoclonal antibodies. Cyclin $D_1$ (**A,** *bottom*), cdk4 (**B,** *bottom*), and cdk6 (**C,** *bottom*) complexes were immunoprecipitated from protein extracts prepared from normal (lanes 1–3) and transgenic mice (lanes 4–7). Kinase activity of the complexes was determined by histone H1 assay.

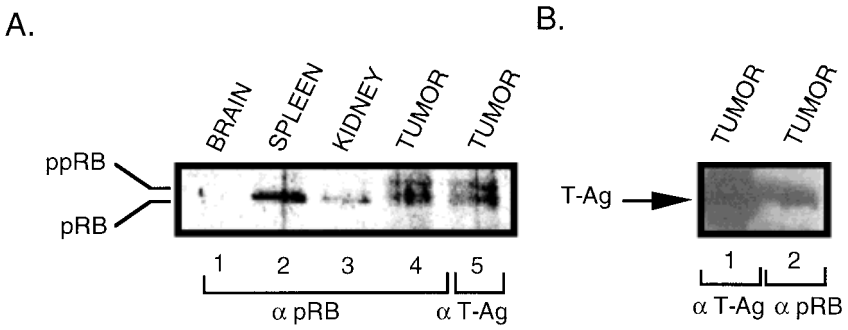


**Fig. 5.** Expression and association of JCV T-antigen with the tumor suppressor protein, pRb. **A:** Proteins extracted from brain, spleen, kidney, and tumor of transgenic mouse were immunoprecipitated with monoclonal antibody against retinoblastoma protein (pRb) (**lanes 1–4**) and with monoclonal antibody against T-antigen (**lane 5**). Immunoprecipitates were fractionated by SDS–PAGE, transferred to nitrocellulose, and subjected to Western blot analysis with a monoclonal antibody against retinoblastoma protein that detects both phosphorylated (ppRb) and its hypophosphorylated (pRb) forms. **B:** Proteins from tumor tissue of transgenic mice were prepared and immunoprecipitated with monoclonal antibody against T-antigen (**lane 1**) or with monoclonal antibody against pRb (**lane 2**). Immunoprecipitates were fractionated by SDS–PAGE, and after transfer to nitrocellulose, incubated with antibody to T-antigen.

In summary, we performed an interrelated series of experiments to evaluate the level of expression and activity of various cell cycle controllers in order to obtain some insight into the molecular pathogenesis of JCV T-antigen-induced CNS tumors in transgenic animals. Based on our observations, we provide a working model as schematized in Figure 7. According to this model, the association of JCV T-antigen with p53 may block the ability of p53 to induce p21WAF1, a protein which inhibits cyclin:cdk activity. De-regulation of the participant cyclins, in particular cyclins E and A, and their associated kinases, may in turn lead to

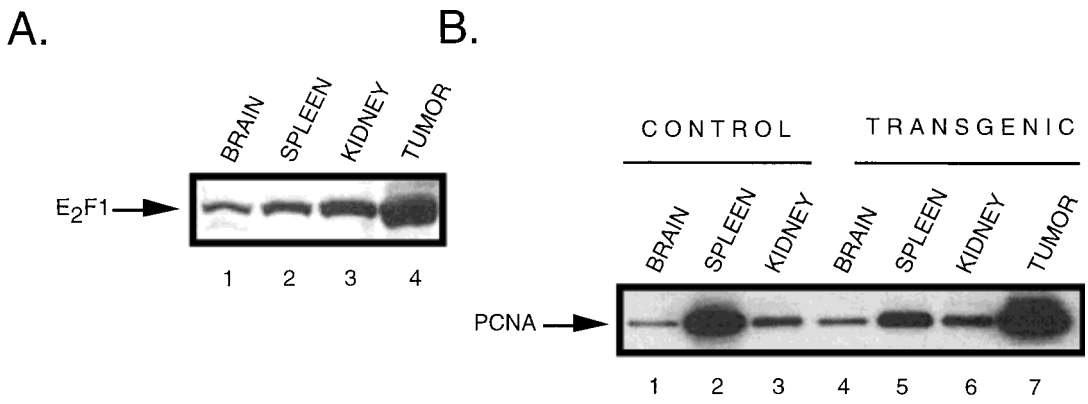


**Fig. 6.** Expression of transcription factor E2F-1 and its responsive gene, PCNA, in experimental animals. **A:** Equal amounts of protein extract (50 µg) isolated from brain, spleen, kidney and tumor of transgenic mice were subjected to Western blot analysis with monoclonal antibody against the transcription factor, E2F-1. **B:** Fifty µg of total protein extracted from various tissues of normal (**lanes 1–3**) and transgenic animals (**lanes 4–7**) were analyzed by Western blotting with antibody that recognizes PCNA.

the phosphorylation of pRb and the liberation of E2F-1. The release of E2F-1 from the pRb–E2F-1 complex may also be accomplished by the association of JCV T-antigen with pRb. As levels of E2F-1 increase in cells, E2F-1 may induce its own gene expression, as well as those from other S-phase-specific promoters such as PCNA, and promote rapid entry of cells into S phase. Perhaps it should be mentioned that none of the transgenic mice created in our laboratory [Franks et al., 1996] and others [Small et al., 1986] developed tumors in brain. The results of this study may also provide important clues as to why JCV T-antigen fails to induce tumors in the brains of experimental animals. For example, elevated levels of p21 in the brains of transgenic mice by a p53-dependent pathway may be an underlying mechanism for the control of cell proliferation and the lack of tumors in brains of these mice. Results from studies on cyclins A and E suggest that despite their comparable levels, complexes associated with these two cyclins in transgenic mouse brain possess more kinase activity than those from control mice. The issue may become even more complicated as their cdk partner, cdk2, which is produced at modest but detectable levels, exhibits no kinase activity. Thus, one may speculate on the involvement of other kinases in association with cyclins A and E in these cells. In light of our data showing high level kinase activity of cyclins A and E, one may anticipate the detection of the phosphorylated form of pRb in tumor cells. It was noted, however, that the increased kinase activity of cyclins A and E in the brain may not correlate with the level and status of pRb in brain cells, as low but detectable levels of pRb in the phosphorylated state were present in brain tissue.

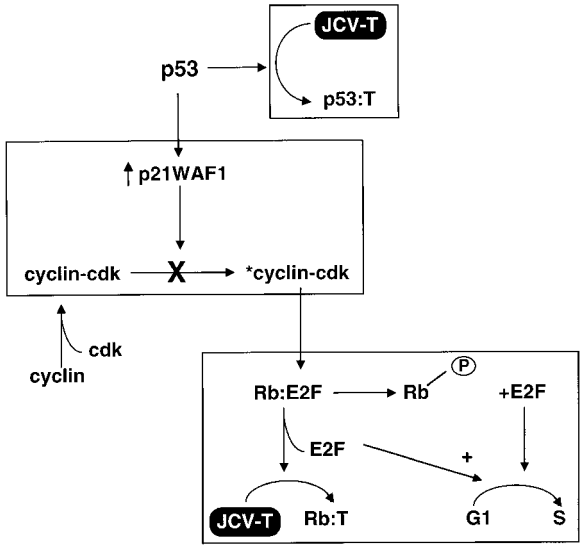


**Fig. 7.** Proposed pathway by which JCV T-antigen induces tumors in experimental animals. Wild type p53 has the capacity to augment transcription of p21WAF-1, an inhibitor of cyclin kinases, including cyclins E and A and their associated kinases. A decrease in kinase activity of cyclin:kinase maintains pRb in a hypophosphorylated state, which in turn sequesters the transcription factor, E2F1. The association of JCV T-antigen with p53 abrogates the ability of p53 to exert its regulatory action via p21WAF-1. In addition, the association of JCV T-antigen with pRb may liberate E2F-1 from pRb:E2F-1 complex and permit E2F-1 to induce transcription of S-phase-specific genes.

Currently we are in the process of evaluating the role of other tumor suppressor proteins which may regulate cyclin activity in tumor and brains of transgenic mice, and are investigating the role of other Rb family members in JCV-induced tumors in transgenic animals.

## ACKNOWLEDGMENTS

We thank members of the Center for NeuroVirology and NeuroOncology for their helpful insight and suggestions and sharing of reagents. We also thank Cynthia Schriver for editorial assistance and Bassel Sawaya for assistance in preparation of figures. This work was made possible by grants awarded by the National Institutes of Health (to K.K.).

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