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JC virus T'_{135}, T'_{136} and T'_{165} proteins interact with cellular p107 and p130 *in vivo* and influence viral transformation potential

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The JC virus (JCV) regulatory proteins, large T antigen, small t antigen, T'_{135}, T'_{136}, and T'_{165}, are encoded by five transcripts alternatively spliced from the viral early precursor mRNA. T antigen and the T′ proteins share N-terminal amino acid sequences that include the L×C×E and J domains, motifs in SV40 T antigen known to mediate binding to the retinoblastoma (Rb) proteins and Hsc70, respectively. In this study, G418-resistant cell lines were created that express wild-type or mutant JCV T antigen and T′ proteins individually or in combination. These cell lines were used to evaluate the ability of each viral protein to bind p107 and p130 *in vivo*, and to influence cellular growth characteristics. Differences were observed in the abilities of individual T′ proteins to bind p107 and p130 and to alter their phosphorylation status. The T′ proteins were also found to localize to the cell's nucleus and to be phosphorylated in a cell cycle-dependent manner. JCV T antigen and T′ proteins expressed from a cytomegalovirus promoter failed to induce dense focus formation in Rat2 cells, but they did cooperate with a mutant Ras protein to overcome cellular senescence and immortalize rat embryo fibroblasts. These data indicate that, despite their sequence similarities, JCV early proteins exhibit unique activities that, in combination, effect the inactivation of cell cycle regulators, a requirement for polyomavirus-induced transformation. *Journal of NeuroVirology* (2006) **12,** 428–442.

Keywords: immortalization; JC virus; phosphorylation; Rb proteins; T′ proteins; transformation

Introduction

The small DNA viruses depend on host cellular factors to replicate their genomes. Viral regulatory proteins, expressed early in an infection, inactivate cell cycle regulators and force the cell's premature entry into S phase, thereby acquiring access to the cell's replication machinery. While progression into S phase is vital to the propagation of the virus in permissive cells, such an event in nonpermissive cells may contribute to oncogenic transformation. JC virus (JCV) is a human polyomavirus that causes transformation of some types of cultured cells and induces tumors upon injection into experimental animals (reviewed in Frisque and White, 1992). Some recent studies have detected the presence of JCV DNA and proteins in a variety of human tumors (reviewed in Eash *et al*, 2006; Niv *et al*, 2005; White and Khalili 2005). It is expected that identifying the mechanisms by which DNA viruses cause cellular transformation will lead to an understanding

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of the molecular events that contribute to human cancer.

Simian virus 40 (SV40), a polyomavirus closely related to JCV, is one of the most widely studied of the small DNA tumor viruses. SV40 expresses three tumor proteins: large T antigen (TAg), small t antigen (tAg), and 17KT (Zerrahn *et al*, 1993). The transforming activities of SV40 TAg are attributed, in part, to a bipartite p53-binding region and to N-terminal L×C×E and J domains, which facilitate binding to the retinoblastoma (Rb) family proteins and to the molecular chaperone protein Hsc70, respectively (Sullivan and Pipas, 2002; Zhu *et al*, 1992). These domains are required to induce cellular proliferation, and to influence events related to programmed cell death. The transformation domains identified in SV40 TAg are conserved in the corresponding positions within the JCV TAg.

The 17KT protein of SV40 shares the first 131 of its 135 amino acids, including the N-terminal functional domains, with the SV40 TAg (Zerrahn *et al*, 1993). 17KT localizes to the cell nucleus, is more abundantly expressed in transformed cells than in lytically infected cells, and has limited transforming ability. Speculation concerning the contribution of 17KT to TAg-induced transformation in the context of wild-type viral DNA revolves around its altered phosphorylation status relative to that of TAg, providing a means for independent regulation of the N-terminal region of TAg.

One of the key steps in viral deregulation of the cell cycle is the inactivation of pRb, a tumor suppressor protein, and the closely related Rb family members, p107 and p130. Tumor proteins expressed by many viruses, including adenovirus E1A protein, human papillomavirus (HPV) E7 protein, and polyomavirus TAgs, share a conserved L×C×E domain, which binds members of the Rb family (DeCaprio *et al*, 1988; Dyson *et al*, 1989; Whyte *et al*, 1988). The Rb proteins are active during the G_0/G_1 phase of the cell cycle, preventing the E2F-dependent transcription of genes required for the cell's progression into S phase. Growth stimulatory signals activate cyclin-dependent kinases (cdk's), which phosphorylate the Rb proteins, leading to the release of E2F family proteins and permitting the transcription of E2F-dependent genes. This mechanism of cell cycle control is superseded by the actions of many viral tumor proteins. Upon binding to the Rb family of proteins, polyomavirus TAgs recruit the molecular chaperone, Hsc70, and stimulate its ATPase activity, promoting the release of E2F (Sheng *et al*, 1997). The interaction between Hsc70 and SV40 TAg is dependent on the TAg J domain (Sullivan *et al*, 2001).

The JCV early regulatory proteins, TAg and tAg, are encoded by transcripts produced via alternative splicing of a single precursor mRNA (Figure 1). These early proteins, in addition to mediating viral replication and influencing viral and cellular transcription in permissive cells (Fanning and Knippers, 1992;

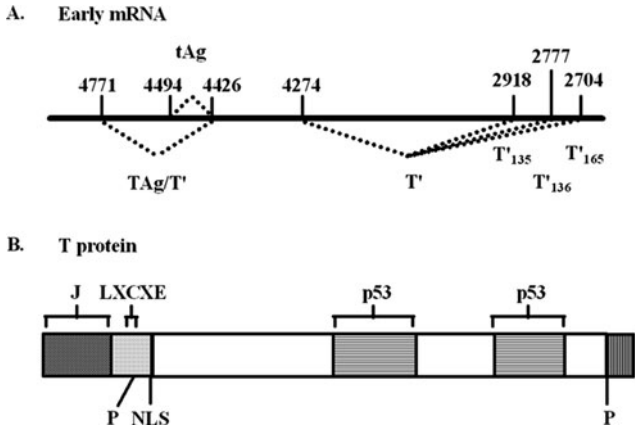

Figure 1 Diagrammatic representation of JCV alternatively spliced early mRNAs and TAg. (**A**) A single precursor mRNA species is alternatively spliced to produce the five JCV early transcripts. Processing of the TAg or tAg transcripts utilizes donor sites at nucleotide (nt) 4771 or 4494, respectively, and a shared acceptor site at nt 4426. Generation of the three T′ transcripts involves the removal of a second intron from the TAg mRNA using a common donor splice site (nt 4274) and one of three unique acceptor sites (nt 2918, T'_{135}; nt 2777, T'_{136}; nt 2704, T'_{165}). The dotted lines indicate the introns removed to produce the specified transcript. (**B**) The functional domains of TAg contributing to viral transformation include the Hsc70-binding J domain (J), amino acids 1 to 81; the Rb-binding domain (L×C×E), amino acids 105 to 109; bipartite p53-binding region (p53), amino acids 351 to 450 and 534 to 628; clusters of N- and C-terminal phosphorylation sites (P); and nuclear localization signal (NLS). Boxes containing cross-hatches, stipples, or vertical lines represent the N-terminal 81 amino acids shared by the five early viral proteins, the region of TAg shared with the three T′ proteins but not tAg (amino acids 82 to 132), or the C-terminal 33 amino acids of TAg shared with T'_{165}, respectively.

Lynch and Frisque, 1991; Sullivan and Pipas, 2002), inactivate cellular proteins that maintain control over cellular proliferation. Trowbridge and Frisque (1995) described three additional JCV splice variants, T'_{135}, T'_{136}, and T'_{165}, originally assumed to be TAg degradation products. The three T′ proteins share their N-terminal 132 amino acids with TAg. T'_{165} also shares its C-terminal 33 amino acids with TAg, whereas the C-termini of T'_{135} and T'_{136} are unique. The sequence identity at the N-termini of the JCV early proteins suggests that these proteins possess overlapping functions (Figure 1). However, the differences at their C-termini are expected to affect their structure and extent of phosphorylation (Scheidtmann *et al*, 1991; Swenson and Frisque, 1995), thereby conferring upon each a unique set of activities. Experimental observations lend support to this hypothesis. First, the N- and C-terminal phosphorylation domains found in TAg are likely retained in T'_{165}, whereas the C-terminal domain is absent in the other two T′ proteins. It has been reported that the initial rate of phosphate turnover of TAg differs from that of T'_{136} (Trowbridge and Frisque, 1995), perhaps reflecting the observation made with the intact SV40 TAg that C-terminal phosphorylation events

influence N-terminal modifications (Scheidtmann *et al*, 1991). Second, the T′ proteins vary in their ability to enhance TAg-mediated DNA replication (Prins and Frisque, 2001). Third, purified TAg and T′ proteins exhibit differential binding *in vitro* to human pRb, p107, and p130, underscoring the possibility that each JCV early protein makes unique contributions to cell cycle progression and transformation (Bollag *et al*, 2000).

The JCV T′ proteins are expressed at much higher levels than the SV40 17KT protein, thus they may have a greater influence on polyomavirus-induced transformation because of more efficient interactions with the Rb proteins. To characterize the *in vivo* binding properties of the JCV early proteins, cell lines stably expressing individual viral proteins were created in the current study. Extracts from these cell lines have been examined to determine the ability of the viral proteins to bind cellular p107 and p130 and to alter their phosphorylation status. In addition, the abilities of the JCV TAg and T′ proteins to induce cellular transformation and to alter cellular growth parameters were investigated.

Results

Derivation of G418-selected Rat2 cell lines expressing individual JCV early proteins

Cell lines expressing individual JCV proteins were derived by cotransfecting Rat2 cells with CMV-JCV cDNA constructs and a plasmid conveying G418 resistance (pSV2-neo) and selecting cells on the basis of survival in the presence of antibiotic rather than on the basis of a transformed phenotype (Trowbridge and Frisque, 1993). With the appearance of G418-resistant ($G418^r$) cell populations, cloned cell lines were generated and screened for viral protein expression by immunoprecipitation and Western blot (IP/WB) analysis. All independent, cloned cell lines expressing the intact early coding region ($R2\text{-}JCV_E$) produced detectable levels of the five early viral proteins. Cell lines expressing T'_{135}, T'_{136}, or T'_{165} ($R2\text{-}T'_{135}$, $R2\text{-}T'_{136}$, and $R2\text{-}T'_{165}$, respectively) were also readily obtained. Cell clones derived following TAg cDNA transfection not only expressed TAg, but also expressed T′ proteins (data not shown). The TAg cDNA retains the splice sites necessary for T′ expression (Figure 1A). To eliminate expression of the three T′ proteins, a mutation was introduced into the TAg cDNA at the shared T′ donor splice site such that the consensus splice sequence was disrupted without altering the amino acid sequence of TAg (see Materials and methods). The G418 selection and cell cloning procedures were repeated with this mutant cDNA construct (pCMV-ΔT′); 30 clones were examined and a single TAg-expressing clone, R2-ΔT′ 1, was obtained. The integrated ΔT′ cDNA was amplified by polymerase chain reaction (PCR) and sequenced to confirm that the donor site mutation (nucleotide [nt] 4274, G → A) was present and that no new mutations had been introduced during establishment of the line. Extracts of the G418-selected cell lines were examined by WB analysis, and those producing similar levels of viral protein were selected for further experimentation (Figure 2).

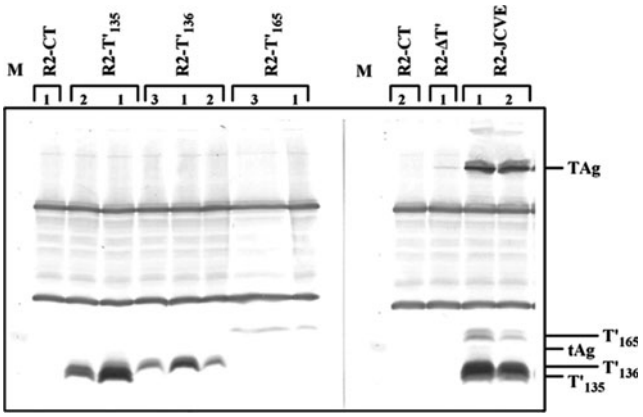

Figure 2 Independent clones of G418-selected cell lines express JCV early proteins. Rat2 lines were examined for the presence and relative amounts of the JCV early proteins. Extracts containing 800 μg total protein (1200 μg for R2-ΔT′ extracts) were immunoprecipitated with PAb 962 and immune complexes were electrophoresed on a 15% SDS-polyacrylamide gel and transferred to nitrocellulose. Western blot analysis was performed using a mixture of PAb 962, 2001, and 2023. The number written below each cell line designation identifies that line as a unique clone. Two R2-CT clones represent $G418^r$ Rat2 control lines that do not produce JCV proteins, while the two $R2\text{-}JCV_E$ clones express all five early viral proteins. A single cell line expresses TAg only (R2-ΔT′), and two or three representative lines express T'_{135}, T'_{136}, or T'_{165}. Protein molecular weight markers (M) were included on both gels used in this composite picture.

T′ proteins are localized to the nucleus

TAg possesses a nuclear localization signal (NLS) sequence near its N-terminus that directs the protein to the cell's nucleus. This signal sequence is within the region of the second exon that is shared by the T′ proteins. The NLS is positioned near the C-termini of the T′ proteins due to the proximity of the signal to the T′ donor splice site. Thus, due to the location of the NLS and the altered protein structures of the T′ proteins, we asked whether the T′ NLS was functional. Antisera, and monoclonal antibodies that recognize different epitopes on the early viral proteins, were used in immunofluorescent staining experiments to visualize the location of the viral protein in each cell line. Distinct nuclear staining was visible in the control JCV-transformed cell line (R2-M1), as well as in cells expressing the intact JCV early coding region ($R2\text{-}JCV_E$) (data not shown). The subcellular location of individual T′ proteins, however, could not be determined by this method. It is possible that the expression level of the T′ proteins in these cells was below that necessary for detection by immunofluorescence. The enhanced green fluorescent protein (EGFP) facilitates the visualization of proteins expressed at low

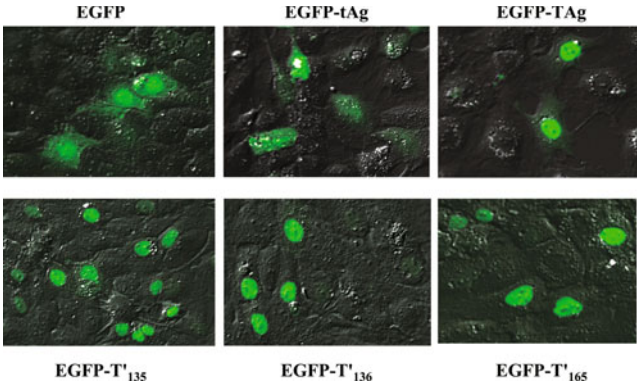

Figure 3 T′ proteins localize to the nucleus. The cDNAs encoding the individual JCV proteins were fused to the EGFP-coding region, and each construct was transfected into Rat2 cells growing on glass coverslips. At 2 days p.t. coverslips were rinsed and examined on an epi-fluorescent microscope, and digital photographs were taken.

levels. Fusion constructs were created by linking the TAg and T′ coding sequences to the 3′ end of the EGFP gene, thereby maintaining the relative position of the T′ protein NLS. Rat2 cells were transfected with the EGFP fusion constructs and photographed after 2 days. Cells expressing EGFP only (empty vector) or EGFP-tAg exhibited diffuse nuclear and cytoplasmic fluorescence, whereas the cells expressing EGFP-ΔT′, EGFP-T′$_{135}$, EGFP-T′$_{136}$, or EGFP-T′$_{165}$ proteins showed nuclear fluorescence only (Figure 3), indicating that the NLS sequence in the T′ proteins is functional.

Individual T′ proteins minimally affect growth properties of G418-selected Rat2 cell lines

We predicted that the growth characteristics of the G418-selected lines would be altered by the expression of one or more of the JCV early proteins. Cell doubling times were determined to evaluate the relative growth rates of several cell lines (Table 1). Independent clones of cell lines expressing TAg, T′$_{135}$, T′$_{136}$, or T′$_{165}$ alone or all five early proteins together (R2-JCV$_E$), were compared to the transformed line R2-M1, and to two G418^r lines that did not express any viral proteins (R2-CT). All of the cell lines exhibited similar rates of growth in medium containing 10% fetal bovine serum (FBS) (Table 1). Saturation densities were also determined for cells propagated in medium supplemented with 1% or 10% FBS concentrations. Most cell lines derived by G418 selection and expressing individual viral proteins exhibited saturation densities in low serum medium that were comparable to those of the R2-CT control lines, although the R2-ΔT′ 1 and R2-T′$_{135}$ 2 cells reached densities that were approximately two-fold higher. The line isolated from a dense focus assay (R2-M1) exhibited cell densities approximately 10-fold higher than that of the R2-CT lines. These transformed cells grew to a slightly higher density in medium supplemented with 10% FBS, whereas the G418-selected lines were influenced to a greater extent by these conditions (about twofold higher densities; data not shown). We also asked which lines might exhibit anchorage independent growth (AIG), one of the most stringent transformation parameters displayed by polyomavirus transformants. The three lines expressing all five JCV early proteins formed visible colonies in this assay. In addition, the R2-ΔT′ 1 and R2-T′$_{135}$ 2 cells grew in soft agarose, with the latter cells forming the largest and most numerous colonies of any line tested. The remaining G418-resistant lines behaved like the control lines and failed to grow under anchorage-independent conditions.

Table 1 Growth phenotypes of cells expressing JCV early proteins

Cell line[a]	*Viral proteins expressed*[b]	*Doubling time (h)*[c]	*Saturation density (×10^{-5})*[d]	*AIG (%)*[e]
R2-M1	T/t/T′$_{135,136,165}$	20	102	13
R2-JCV$_E$ 1	T/t/T′$_{135,136,165}$	20	10	1
R2-JCV$_E$ 2	T/t/T′$_{135,136,165}$	21	10	4
R2-ΔT′ 1	T	21	19	1
R2-T′$_{135}$ 1	T′$_{135}$	23	5	<0.03
R2-T′$_{135}$ 2	T′$_{135}$	23	18	19
R2-T′$_{136}$ 1	T′$_{136}$	20	7	<0.03
R2-T′$_{136}$ 2	T′$_{136}$	22	6	<0.03
R2-T′$_{136}$ 3	T′$_{136}$	ND	ND	<0.03
R2-T′$_{165}$ 1	T′$_{165}$	20	8	<0.03
R2-T′$_{165}$ 2	T′$_{165}$	24	7	ND
R2-T′$_{165}$ 3	T′$_{165}$	20	12	<0.03
R2-CT 1	None	21	7	<0.03
R2-CT 2	None	25	10	<0.03

[a]All Rat2 (R2) cell lines were derived by G418 selection except for R2-M1, a cloned transformed line obtained in a dense focus assay (Bollag *et al*, 1989). The last number in the cell line name denotes an independent clone.
[b]Cell lines express either 0 (none), 1 or all 5 JCV early proteins: TAg (T), tAg (t), T′$_{135}$, T′$_{136}$, or T′$_{165}$.
[c]Doubling times were calculated at different time intervals using the formula $dt = t(\ln 2)/\ln(c_{t2}/c_{t1})$, where t = time (h) between cell counts, c_{t1} and c_{t2} = counts at the first and second time points, respectively. Shown are doubling times calculated from cell counts at 2 and 6 days (2 and 8 days for R2-ΔT′ 1) after cell plating. ND, not determined.
[d]At day 35 post plating, saturation densities were measured for cells growing in duplicate 60-mm plates containing DMEM supplemented with 1% FBS.
[e]Anchorage independent growth (AIG) was determined 21 days after plating cells in soft agarose containing DMEM supplemented with 10% FBS. Colonies with diameters exceeding 0.05 mm were counted in 30 fields (1.96 mm^2/field) selected at random. A value of <0.03% indicates no colonies were observed in any of the fields.

JCV T′ proteins bind differentially to the retinoblastoma proteins p107 and p130 in vivo

Functional inactivation of the Rb family of tumor suppressor proteins is an early step in cellular transformation induced by small DNA tumor viruses. Binding to the Rb proteins requires the L×C×E domain present in many viral tumor proteins. The J domain, found at the N-terminus of polyomavirus tumor proteins, facilitates binding to the cellular chaperone protein Hsc70 and is required for inactivation of

the Rb family of proteins. Because the JCV T′ proteins possess the L×C×E domain, we asked whether they were able to bind the Rb proteins *in vivo*. Extracts were prepared from each G418-selected cell line, and cellular Rb or viral T proteins were immunoprecipitated with the appropriate antibodies. Cells transfected with the G418^r gene alone (R2-CT) were used as a negative control. Interactions with pRb were difficult to detect in these experiments presumably because of low levels of pRb in Rat2 cells (data not shown; Young and Longmore, 2004), but binding to p107 or p130 by JCV early viral proteins was visualized by this approach. Lines expressing individual JCV proteins at levels similar to that observed in R2-JCV$_E$ cells were chosen for analysis (Figure 4A). R2-ΔT′ cells represented an exception, because the single line available for testing produced

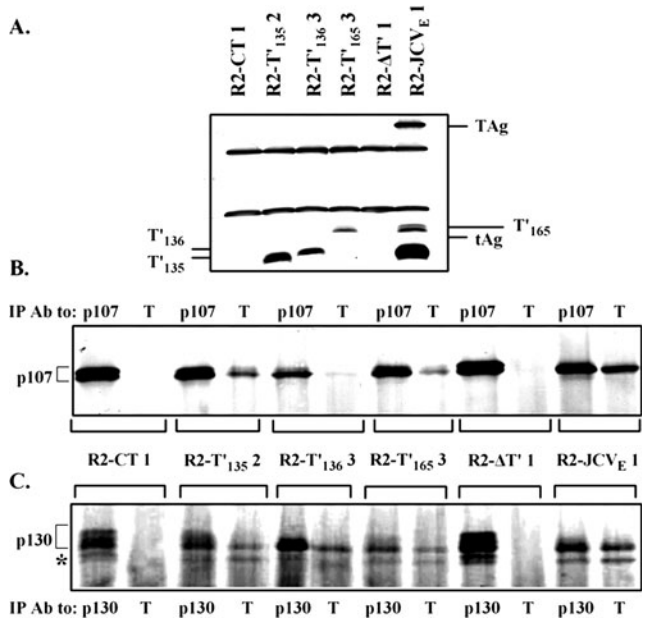

Figure 4 JCV early proteins interact with the Rb family proteins p107 and p130. (**A**) Relative amounts of JCV early proteins in extracts of cells used in parts **B** and **C**. Cell clone numbers are indicated. Samples (1 mg total protein) were immunoprecipitated with PAb 962, and the proteins were separated on a 15% SDS-polyacrylamide gel. Western blot analysis was performed using a mixture of PAb 962, 2001, and 2023. (**B**) Interactions between JCV early proteins and p107. Samples of 1.5 and 1.0 mg total protein were immunoprecipitated with PAb 962 and anti-p107 antibodies, respectively, and the proteins were separated on a 6% SDS-polyacrylamide gel. Western blot analysis was performed using anti-p107 antibody. The amount and phosphorylation status of p107 present in each cell line, as well as the amount and modified species bound to the JCV early proteins, was observed. (**C**) Interactions between JCV early proteins and p130. Samples of 3.0 and 2.0 mg total protein were immunoprecipitated with PAb 962 and anti-p130 antibodies, respectively, and the proteins were separated on a 6% SDS-polyacrylamide gel. Western blot analysis was performed using anti-p130 antibody. The amount and phosphorylation status of p130 present in each cell line, as well as the amount and modified species bound to the JCV early proteins, was observed. The asterisk (*) denotes p107, which is recognized by the commercial anti-p130 antibody used in the WB.

TAg in very low amounts. Initially, IP/WB assays were performed with anti-p107 or anti-p130 antibodies to compare the phosphorylation status and amounts of the endogenous p107 and p130 proteins in naïve or JCV-containing cells. The cellular proteins exist as differentially phosphorylated species in R2-CT cells, apparent as two or more bands on SDS-polyacrylamide gels (Figure 4B, C, lane 1). Cells expressing TAg alone also had multiple species of p107 and p130 (Figure 4B, C, lane 9), whereas cells expressing T′$_{135}$, T′$_{136,}$ or T′$_{165}$ contained reduced amounts of the hyperphosphorylated forms of these Rb family members (Figure 4B, C, lanes 3, 5, 7). The expression of the intact JCV early region nearly eliminated the hyperphosphorylated species of p107 and p130 (Figure 4B, C, lane 11).

To detect physical interactions between the viral and cellular proteins, anti-T antibodies were employed in the IP step, and anti-p107 or -p130 antibodies were used in the WB step. The co-IP/WB assay revealed that T′$_{135}$ and T′$_{165}$ bound the hypophosphorylated species of p107 (Figure 4B, lanes 4, 8). An interaction between T′$_{136}$ and p107 also occurred but was difficult to detect (Figure 4B, lane 6); extracts from two other independent clones of R2-T′$_{136}$ cells yielded the same result (data not shown). Interestingly, even in the apparent absence of efficient binding, the levels and phosphorylation status of p107 were clearly reduced in R2-T′$_{136}$ cells (Figure 4B, lane 5). The greatest amount of p107 coimmunoprecipitated in these assays occurred using extracts of R2-JCV$_E$ cells, whereas no detectable interaction was noted using the R2-ΔT′ extracts (Figure 4B, lanes 10, 12). The latter observation likely reflects the low amount of TAg present in this line. Complex formation was also detected between the hypophosphorylated forms of p130 and the JCV early proteins (including T′$_{136}$) in each cell line except the R2-ΔT′ line (Figure 4C, lanes 4, 6, 8, 10, 12).

Derivation of Rat2 cell lines expressing H42Q and E109K mutant proteins

The L×C×E and J domains of SV40 TAg are required for its interaction with Rb family members and for release of E2F from the Rb-E2F complex (Zalvide *et al*, 1998; Harris *et al*, 1998). To confirm the expectation that the J and L×C×E domains of JCV early proteins contribute to their interactions with Rb proteins, mutations were introduced into these two motifs to yield the H42Q and E109K mutants, respectively. The mutated DNAs were transfected into Rat2 cells to establish stable G418^r lines, and cells expressing viral proteins at levels similar to those detected in R2-JCV$_E$ cells were selected for further analysis. The ability of the mutant JCV proteins to bind p107 and p130 *in vivo* was examined using extracts of the R2-H42Q and R2-E109K cell lines and the co-IP/WB approach (Figure 5). Complex formation between JCV early proteins and p107 and p130 was observed in extracts of R2-JCV$_E$ and R2-H42Q cells, but not in

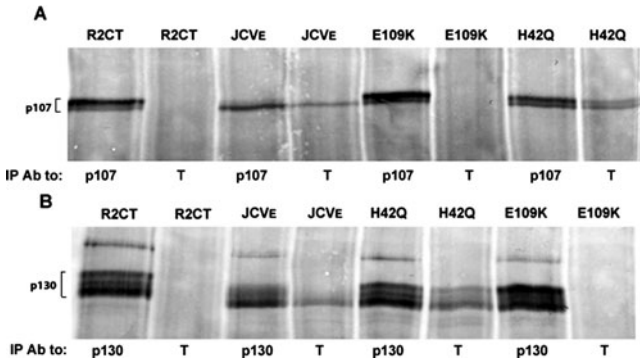

Figure 5 Ability of J and L×C×E domain mutants of JCV early proteins to bind p107 and p130. Proteins in cell extracts prepared from R2-CT, R2-JCV_E, R2-E109K, and R2-H42Q cells were immunoprecipitated with either PAb 962, anti-p107, or anti-p130 antibody and separated on a 6% SDS-polyacrylamide gel. Western blot analysis was performed using either anti-p107 (**A**) or anti-p130 (**B**) antibody.

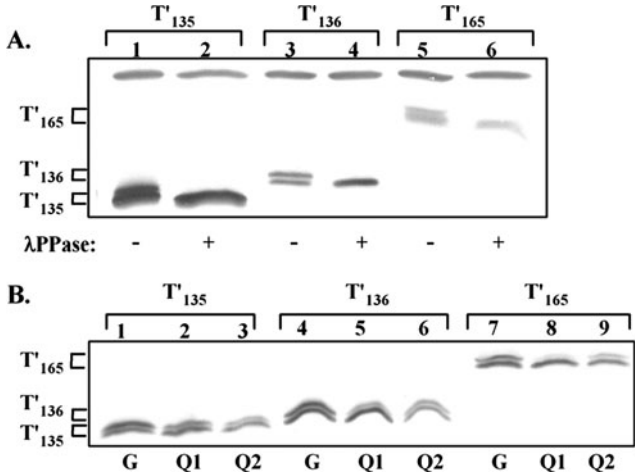

Figure 6 Phosphorylation status of JCV T′ proteins differs in growing versus quiescent cells. (**A**) Immunoprecipitated extracts from G418-selected cell lines expressing individual T′ proteins were treated with λ-PPase and compared to untreated samples to confirm the phosphorylation status of each T′ protein. Proteins from R2-T'_{135} 1 (400 μg), R2-T'_{136} 3 (800 μg) and R2T'_{165} 3 (900 μg) cells were immunoprecipitated with PAb 962. Immune complexes were incubated with λ-PPase in reaction buffer or with reaction buffer alone, and proteins were separated on a 20% SDS-polyacrylamide gel. Western blot analysis was performed using a mixture of PAb 962, 2001, and 2023. (**B**) Extracts from actively growing cells (G) and cells quiescent for 1 (Q1) or 2 (Q2) days were analyzed to determine the phosphorylation state of each protein at different stages of growth. Proteins were immunoprecipitated from R2-T'_{135} 2 (400 μg), R2-T'_{136} 2 (1500 μg) and R2-T'_{165} 3 (900 μg) cells with PAb 962 and separated on a 20% SDS-polyacrylamide gel. Western blot analysis was performed using a mixture of PAb 962, 2001, and 2023.

extracts of R2-E109K cells. In each extract the viral proteins associated with only a fraction of the total pool of p107 or p130. Further, wild-type JCV proteins in R2-JCV_E cells interacted only with the faster migrating form(s) of p107 and p130 (Figure 5A, B, lane 4), whereas the J domain mutant proteins in R2-H42Q cells bound to both the slower and faster migrating forms of p107 and p130 (Figure 5A, lane 8; Figure 5B, lane 6). The L×C×E domain mutant proteins failed to interact with, or to alter the phosphorylation state of, the two Rb family members (Figure 5A, lanes 5, 6; Figure 5B, lanes 7, 8). These observations indicate that interactions of JCV TAg and T′ proteins with p107 and p130 require the L×C×E domain, whereas the degradation or dephosphorylation of hyperphosphorylated p107 and p130 requires the J domain.

Phosphorylation of T′ proteins in growing vs. quiescent cells

T′ proteins present in our cloned G418-selected cell lines varied in terms of phosphorylation status and level of expression depending on the confluence of the cells at the time cell extracts were prepared. We first confirmed that the doublet bands seen in immunoprecipitated extracts of T′-expressing cells represented differentially phosphorylated forms of the T′ proteins. Immunoprecipitated samples prepared in parallel were treated with λ-phosphatase or left untreated and the migration patterns of the resulting proteins on polyacrylamide gels were compared (Figure 6A). The treated samples contain a single form of each T′ protein, indicating that the slower migrating form in the untreated samples is phosphorylated. To investigate the possibility that expression and phosphorylation of T′ proteins are dependent upon the growth status of the cells, extracts were made from cells that were either actively growing or maintained at confluence under low serum conditions for 1 or 2 days. An IP/WB assay was performed on each set of extracts and the protein bands were compared. The actively growing population of each cell line possessed two distinct forms of the T′ proteins, a hyperphosphorylated and a hypophosphorylated species (Figure 6B, lanes 1, 4, 7). Upon reaching confluence, the levels of hyperphosphorylated T'_{136} and T'_{165} diminished, but began to reappear on day 2 (Figure 6B, lanes 5 versus 6 and 8 versus 9). The levels of hypophosphorylated T'_{165} appear to remain constant while the overall levels of T'_{136} appear to decrease. In contrast, the levels of both forms of T'_{135} appear to decrease as the cells become quiescent (Figure 6B, lanes 1, 2, 3).

Transforming activity of JCV early proteins

The JCV T′ proteins are predicted to influence viral transformation efficiency based on the presence of two TAg transformation domains, the Rb-binding L×C×E domain, and the Hsc70-binding J domain (Figure 1B). To assess the transformation potential of the T′ proteins, Rat2 cells were transfected with constructs encoding individual JCV proteins, and a dense focus assay was performed to assess the loss of contact inhibition characteristic of transformed cells. The intact JCV genome transforms rodent fibroblasts inefficiently, presumably due to viral protein

expression levels below that required to induce transformation (Haggerty *et al*, 1989; Trowbridge and Frisque, 1993). Only rarely are transformants, such as R2-M1 (Bollag *et al*, 1989), isolated by this method. To achieve the viral protein expression levels necessary for transformation, the early region may be placed under the control of a strong promoter, such as that from SV40 or cytomegalovirus (CMV). In the present study, the coding sequences for TAg and each T′ protein were linked to the CMV early promoter, and these constructs were transfected into the Rat2 line. Using this approach we determined that individual JCV early proteins were unable to induce Rat2 cells to adopt a dense focus phenotype (data not shown). As a second approach to demonstrate transforming activity of the JCV T′ proteins, we performed Ras cooperation experiments. Prolonged exposure of primary cells to Ras accelerates the process of cellular senescence, and expression of full-length SV40 TAg or an N-terminal truncated TAg bypasses this growth arrest. To test the ability of CMV-JCV cDNA constructs to provide immortalizing activity and fully transform rat embryo fibroblasts (REF) in combination with a *ras* oncogene, REF were cotransfected with the H-*ras* vector, Sp72-ras, and one or more constructs encoding the JCV and SV40 early proteins. The results from three independent experiments are shown in Table 2. As expected the SV40 control (pPVUO) yielded about twice as many foci as the intact JCV early region (JCV_E); SV40 17KT was also shown for the first time to cooperate with Ras to form dense foci and immortalize cells. In agreement with data presented in Table 1, T'_{135} was more active than the other JCV early proteins. Dense foci induced by each DNA were isolated, expanded in 35-mm plates and passaged at ratios of 1:100; cells still proliferating after eight passages (>70 doublings) were considered immortalized. The majority of foci induced by JCV TAg alone senesced after three or four passages (similar to *ras* only controls). One half of the lines generated with T'_{165} became immortal; similar numbers were seen with the T'_{165} plus TAg cotransfection. Nearly all lines derived from dense foci and expressing JCV T'_{135}, T'_{136}, SV40 17KT, or intact JCV or SV40 early regions were immortalized. Senescent cells, including those transfected with salmon sperm, Sp72-ras, or CMV-ΔT′ DNA, were readily distinguishable from the immortalized cells on the basis of their larger size and flattened appearance (Figure 7). WB analysis of extracts of the REF lines revealed higher and more consistent levels of JCV early protein expression relative to that observed in the G418-selected Rat2 lines (Figure 8A). It was noted that TAg was present as two bands in one clone each of the REF-JCV_E and REF-ΔT′ cells, but as a single band in the second clone of these lines. The three T′ proteins migrated as

Table 2 JCV early proteins cooperate with H-ras to transform REF

	Dense focus formation, % of JCVE[b]			*Immortalization*[c]
Construct + H-ras[a]	*Experiment 1*	*Experiment 2*	*Experiment 3*	*Experiment 2*
CMV-JCVE	100 (61)	100 (28)	100 (59)	24/24
CMV-JCT	ND	ND	66	ND
CMV-ΔT′	28	39	78	2/18
CMV-ΔT′+ CMV-T'_{165}	ND	61	ND	15/24
CMV-T'_{165}	89	68	71	12/24
CMV-T'_{136}	49	57	122	21/22
CMV-T'_{135}	96	182	86	20/22
pPVU0 (SV40 TAg, tAg, 17KT)	139	207	125	12/12
17KT intron (SV40 17KT)	ND	54	ND	23/23
Sp72-ras only	0	2	6	0/2
SS DNA	0	0	ND	0/2
CMV-JCVE, no *ras*	0	ND	ND	ND
CMV-T′, no *ras*	ND	ND	0	ND
pPVU0 (SV40 TAg, tAg, 17KT), no *ras*	0	ND	ND	ND

[a]REF were cotransfected with a plasmid(s) expressing 1 or more JCV or SV40 early proteins and the H-*ras* expressing vector, Sp72-ras. The CMV-JCT construct produces JCV TAg, T'_{135}, T'_{136}, and T'_{165}, the pPVU0 vector produces SV40 TAg, tAg and 17 KT and the 17KT intron vector expresses SV40 17 KT alone. Negative controls included cells transfected with SS (salmon sperm) DNA + Sp72-ras, Sp72-ras alone, and either CMV-JCVE, CMV-T'_{135}, or pPVU0 alone. Although pPVU0 will transform REF independently, dense foci will not appear in the time frame of the experiment in the absence of Ras. ND, not determined.

[b]Cells were stained with crystal violet between days 11 and 18 p.t. Foci were detected first on plates transfected with CMV-JCVE or pPUV0 and rapidly grew in circumference and density. Foci arose slowly on CMV-ΔT′ plates which were stained at the same time as the Sp72-ras only plates. Foci on these two sets of plates remained small and did not pile up in dense layers. The average number of foci counted per plate for each tested DNA is calculated as a percentage of that determined for CMV-JCVE (the average number of foci per plate is shown in parentheses and was assigned a value of 100%).

[c]Immortalizing activity was assessed during Ras cooperation experiment 2 by isolating cells from dense foci prior to crystal violet staining, expanding them in 35-mm dishes, and passaging them at a ratio of 1:100 upon reaching confluence. A line was considered immortal if the cells grew beyond passage 8 (>70 doublings); the number of immortalized clones per total number of foci tested is shown. All foci visible on the unstained CMV-ΔT′ (18) and Sp72-ras only (2) plates were isolated and tested. Foci did not form on the SS DNA plates, but cells were removed randomly from the plates and served as controls. The number of foci present on the other transfection plates was not a limiting factor when deciding upon the number of foci to isolate for the experiment.

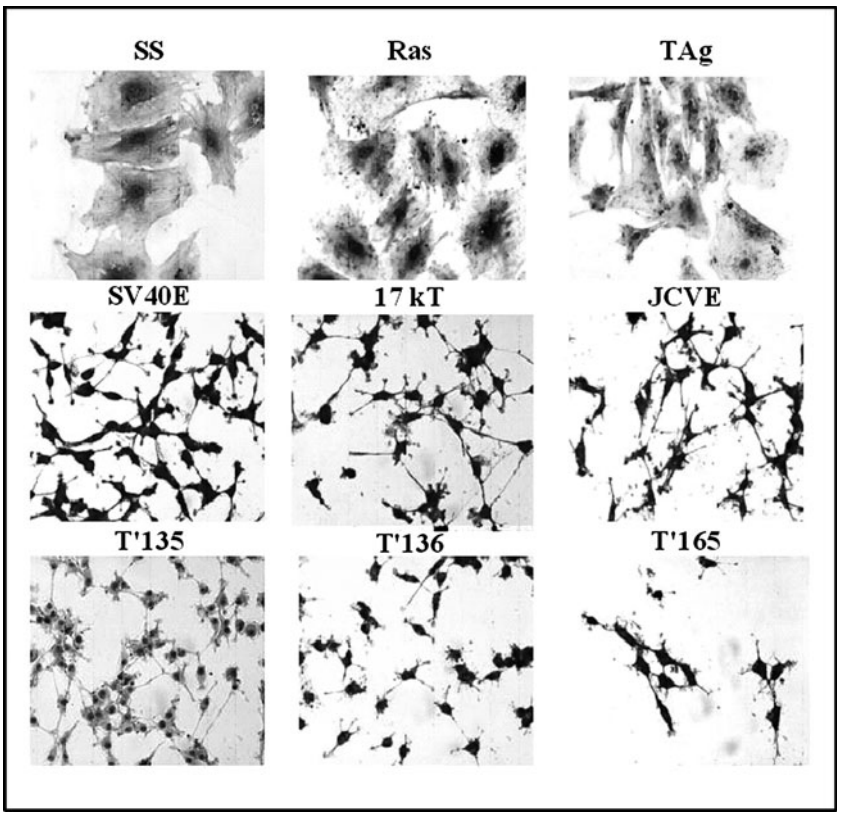

Figure 7 Morphology of cells isolated from the Ras cooperation assay. Cells were plated sparsely on 60-mm dishes and approximately 24 h later were fixed and stained with a crystal violet solution. Photomicrographs of all cells shown were taken using the same magnification (400×) to record cellular morphology. Cells transfected with salmon sperm (SS), Sp72-ras (Ras), or pCMV-ΔT′ (TAg) DNA were photographed at passage 2 as they approached senescence. Cells expressing all 5 JCV (JCVE) or 3 SV40 (SV40E) early proteins, or individual SV40 (17KT) or JCV (T'_{135}, T'_{136}, T'_{165}) TAg splice variants were photographed at passage 8 or 9.

doublets in each of the cloned lines. Because high levels of viral protein were detected in these cells, we reexamined the interactions of JCV TAg and T'_{136} with cellular p107, and p130; in these experiments the co-IP step employed either anti-T, -p107, or -p130 antibodies and the WB step was performed using a mixture of anti-T antibodies (Figure 8B). This approach allowed us to readily demonstrate that the two viral proteins bind to both Rb family members, and further, that both hyperphosphorylated and hypophosphorylated species of each T′ protein interact with p107 and p130. In addition, we observed a very weak band in the two REF-ΔT′ clones that was recognized by the anti-T monoclonal antibodies and migrated at the position of T'_{136} (Figure 8A). Finally, a faint band was reproducibly detected in JCV_E extracts that migrated at the position of tAg and interacted with p130 (Figure 8B, lane 3).

Discussion

It is well established that the SV40 TAg N-terminal domains are required for cellular transformation. Although the JCV TAg shares these N-terminal domains, JCV transforms fewer cell types in culture, and with lower efficiency than does SV40. Given this restricted transforming behavior of JCV, initially we decided to generate cell lines expressing wild-type and mutant JCV early proteins using a G418-selection approach. These cell lines were then utilized to examine transformation-related functions of JCV TAg and the three T′ proteins. Subsequently, we attempted to investigate the transforming potentials of JCV TAg and T′ proteins directly using a dense focus assay in the Rat2 cell line. As we had anticipated, this approach was unsuccessful. However a second approach conducted in primary REF indicated that JCV T′ proteins are capable of inducing an immortalized, transformed phenotype in cooperation with mutant Ras.

Multiple cell lines expressing all five JCV early proteins together or individual T′ proteins alone were derived following G418 selection of transfected Rat2 cells. Surprisingly, after several attempts in both Rat2 cells and mouse NIH-3T3 cells (data not shown), we were able to generate just one Rat2 line expressing JCV TAg alone, and in these cells the level of TAg expression was very low. We also had difficulty obtaining immortalized REF using the CMV-ΔT′ construct, although two lines were derived, and they expressed substantial amounts of TAg. One interpretation of these results is that TAg has a negative effect on

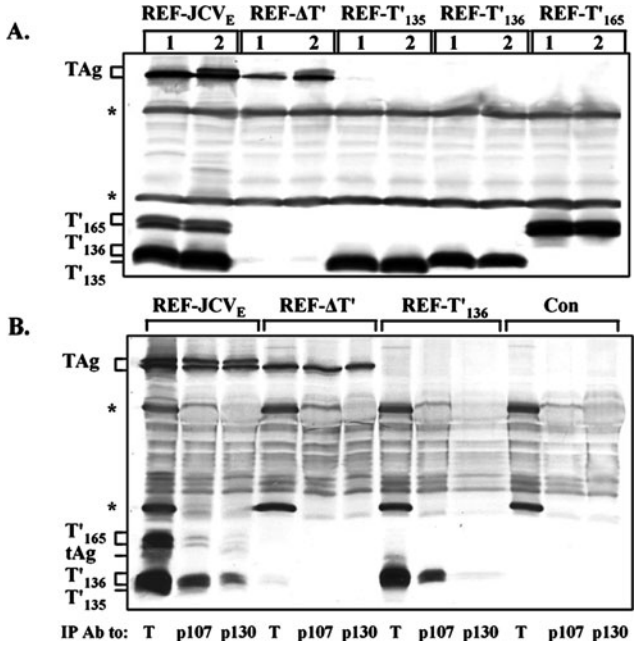

Figure 8 JCV TAg and T′$_{136}$ bind p107 and p130 in extracts of immortalized REF. (**A**) Relative amounts of JCV early proteins in extracts of cells examined in part **B**. Results for two independent clones of each REF cell line are shown. Samples (1 mg total protein) were immunoprecipitated with PAb 962, and the proteins were separated on a 15% SDS-polyacrylamide gel. Western blot analysis was performed using a mixture of PAb 962, 2001, and 2023. The asterisks (*) denote IgG heavy and light chains. (**B**) Interactions between JCV early proteins and cellular p107 and p130 were examined as described in the legend to Figure 4, except the Western blot step was performed with anti-T antibodies. Extracts of cell lines REF-JCV$_E$ 2, REF-ΔT′ 1, and REF-T′$_{136}$ 1 were prepared. Samples of 0.35, 3.5, and 7.0 mg total protein were immunoprecipitated with PAb 962, anti-p107, or anti-p130 antibody, respectively, and the proteins were separated on a 15% SDS-polyacrylamide gel. Western blot analysis was performed using a mixture of PAb 901, 962, 2000, 2001, 2003, 2023, 2024, and 2030. The identity and phosphorylation status of the JCV early proteins bound to the host proteins were determined. Control (Con) lanes contain EBC buffer treated with the three different antibodies used in the immunoprecipitation step. The asterisks (*) denote IgG heavy and light chains.

rodent cell proliferation or survival, which is blocked by Ras or the other JCV early proteins. Our observation that T′$_{136}$ might be produced at very low levels in REF-ΔT′ (Figure 8) and R2-ΔT′ (data not shown) cells would support this interpretation, but it would also indicate that the T′ donor site mutation in our CMV-ΔT′ construct is leaky, an observation made by Boyapati *et al* (2003) when trying to generate a SV40 17KT null mutant using a similar approach.

From previous studies (Trowbridge and Frisque, 1993), we expected to find considerable variation in JCV protein expression levels in independent cell clones, and that induction of a transformed phenotype would be influenced by the amount of viral protein produced. Based upon our IP/WB data, we selected G418^r Rat2 lines for analysis that contained similar levels of each JCV protein. Rodent cells exposed to primate polyomaviruses may acquire a number of new growth properties that signal a transformation event. We compared our G418-selected cells to a JCV-transformed line selected previously from a dense focus assay (R2-M1) to determine if the presence of one or more JCV early proteins decreased their doubling times and increased their saturation densities and AIG potential. The latter property is considered one of the most stringent transformation criteria for polyomavirus transformed cells, and the three lines expressing all five JCV early proteins (R2-M1, R2-JCV$_E$ 1 and 2) formed colonies in soft agarose. Only two additional lines exhibited AIG, R2-ΔT′ and R2-T′$_{135}$ 2, and these same cells reached the highest saturation densities of the G418^r lines. The third characteristic tested, doubling time, did not permit us to readily distinguish between control and JCV-expressing cell lines. Except for R2-T′$_{135}$ 2 cells which appeared to be fully transformed in our assays, lines containing individual T′ proteins exhibited growth parameters consistent with those observed in minimal transformants (Risser and Pollack, 1974). It should be noted that the second cloned R2-T′$_{135}$ line (clone 1) expressed much higher levels of T′$_{135}$ than did clone 2, but the cells failed to grow in soft agarose or to high saturation density. It is possible that T′$_{135}$ levels above a certain threshold limit are detrimental to the protein's ability to induce a transformed phenotype and this phenomenon is under investigation. The transformation behavior (AIG) exhibited by the single TAg-expressing line was unexpected given the low amounts of TAg present in R2-ΔT′ cells and our inability to detect efficient TAg-Rb interactions in the cell extracts. It is possible that our successful derivation of the single R2-ΔT′ line was the result of selecting rapidly dividing cells that expressed TAg at a level capable of inducing a transformed phenotype in the absence of adverse effects on cell survival.

The JCV T′ proteins interact with members of the Rb tumor suppressor family and confer a partially transformed phenotype upon G418-selected cells expressing these viral proteins. However, direct evidence implicating individual JCV proteins in the transformation process was not available until Ras cooperation experiments were performed. Prolonged exposure of primary rodent cells to the *ras* oncogene leads to the activation of p53 and p16 pathways, inhibiting cell cycle progression and leading to cellular senescence (reviewed in Beachy *et al*, 2002). Ras-induced senescence of primary cells is overcome by expression of either N- or C-terminal sequences of SV40 TAg that promote immortalization and a transformed phenotype. As predicted, based on the activity of SV40 TAg fragments (Beachy *et al*, 2002; Cavender *et al*, 1995), SV40 17KT was found to exhibit the Ras cooperation phenotype even though this protein lacks p53-binding capability. Similarly, individual JCV early proteins possess this activity, and expression of T′$_{135}$ or T′$_{136}$ proteins appears to have a

greater influence in this assay than does the expression of TAg and T'_{165}. In the latter case, approximately one half of the lines generated with T'_{165} became immortal. Similar numbers were seen when T'_{165} and TAg were coexpressed, suggesting that transforming activity of this T′ protein is dominant over the potential deleterious effect that TAg has on cell proliferation.

The L×C×E and J domains of polyomavirus tumor proteins play key roles in deregulating the cell cycle and promoting transformation of nonpermissive cells. IP/WB experiments confirmed that the JCV T′ proteins bind Rb proteins and influence their phosphorylation status. The three T′ proteins share their N-terminal amino acids and have short unique C-terminal ends; they are essentially L×C×E and J domains linked to a functional NLS. It is also possible that within the T′ protein coding regions a third sequence motif exists that binds the ubiquitin ligase Cul7 and influences transforming behavior (Ali *et al*, 2004; Kasper *et al*, 2005; Kohrman and Imperiale, 1992). T'_{135} and T'_{136} are especially closely related, differing only in their three or four C-terminal amino acids, respectively. Despite the similarity of sequence, the two proteins exhibit functional differences. In the present study it was difficult to demonstrate an interaction between T'_{136} and p107, however, p107 levels and hyperphosphorylated species were reduced to an even greater extent in R2-T'_{136} cells than in cells producing other JCV early proteins. It is possible that upon formation of a complex, T'_{136} promotes rapid degradation of p107, thereby disrupting the interaction prior to its detection. All three T′ proteins interacted with the hypophosphorylated species of p130, but once again T'_{136} appeared to have the greatest effect on the phosphorylation status of p130. Our findings support those of others who have reported that truncated SV40 TAg or T′-like proteins such as SV40 17KT promote degradation and/or dephosphorylation of Rb proteins (Boyapati *et al*, 2003; Lin and DeCaprio, 2003, Stubdal *et al*, 1996).

To confirm that the L×C×E and J domains of JCV proteins influence their ability to regulate Rb functions, extracts of cell lines stably expressing E109K or H42Q mutant proteins were examined with the IP/WB assay. Relative to R2-JCV_E cells, R2-E109K cells contain species of p107 and p130 that migrate slower on polyacrylamide gels, suggesting that JCV proteins with an altered L×C×E motif fail to eliminate the hyperphosphorylated form(s) of p107 and p130. In addition, direct binding of E109K proteins to p107 and p130 was not observed. The role of the J domain appears to be to recruit the co-chaperone protein, Hsc70, to the Rb-E2F complex and activate its intrinsic ATPase activity, thereby disrupting the complex and releasing E2F (Sheng *et al*, 1997; Sullivan *et al*, 2000). JCV H42Q mutant proteins are able to interact with both the hyperphosphorylated and hypophosphorylated forms of p107 and p130, but fail to reduce the levels of hyperphosphorylated p107 and p130 in the R2-H42Q line. As reported earlier for SV40 TAg (Boyapati *et al*, 2003; Stubdal *et al*, 1997), our findings suggest that the J domain of JCV tumor proteins contributes to the disappearance of hyperphosphorylated forms of p107 and p130. The wild-type and H42Q mutant proteins appeared to differ in their ability to interact with hyperphosphorylated p107 and p130. To determine whether this observation was simply due to the absence of hyperphosphorylated forms in R2-JCV_E cells, or because wild-type proteins are unable to bind these forms even if present, JCV early proteins were incubated with extracts of the parental Rat2 cells. The results revealed that even when hyperphosphorylated forms were available in the extract, JCV wild-type early proteins, unlike J domain mutant proteins, bind only the faster migrating, underphosphorylated species of p107 and p130 (data not shown).

The experiments with the J and L×C×E mutants confirm that the latter domain is required for the binding of JCV TAg and T′ proteins to Rb proteins. However, co-IP/WB experiments in REF-JCV_E cells, which produce high levels of all five JCV early proteins, led to the discovery of an interaction between a 20-kDa protein recognized by the anti-T monoclonal antibody and p130 (but not p107). If this band represents JCV tAg, which lacks the L×C×E domain, this observation would indicate that another motif in the unique C-terminus of tAg binds p130. Currently there are no reports of a polyomavirus tAg binding to an Rb protein. It is also possible that the 20-kDa band represents a previously undiscovered JCV T′ protein that retains the J and L×C×E domains. An earlier mutagenesis study (Prins and Frisque, 2001) identified new T′ transcripts that might be produced using cryptic splice sites in the JCV precursor mRNA. Work is underway to determine the identity of this protein.

IP/WB assays utilizing R2ΔT′ extracts did not reveal an interaction between TAg and p107 or p130. However, weak binding of TAg to these Rb family members was observed when we repeated the assay using the more sensitive chemiluminescence detection scheme (data not shown). We suggest that these data simply reflect the low level of TAg present in this cell line. Analysis of cell lines generated in our Ras cooperation experiments supports this idea; at higher levels of TAg expression in REF, interactions are readily demonstrated. It should be noted, however, that binding to p107 and p130 is equivalent in cells expressing either all five JCV early proteins or only the three T′ proteins (Tyagarajan and Frisque, 2006), suggesting the possibility that under normal conditions, T′ proteins may free TAg to interact with its other multiple cellular protein partners.

The inactivation of Rb proteins and the release of E2F require an intact J domain, which mediates binding to Hsc70 (Zalvide *et al*, 1998). Sullivan and coworkers (2001) were unable to demonstrate an interaction between Hsc70 and a truncated

form of SV40 TAg (N136), and they concluded that C-terminal sequences might cooperate with the J domain to effect stable binding. However, it is possible that truncated forms of TAg do interact transiently with Hsc70 and that such interactions facilitate J domain function. For example, the SV40 N-terminal fragment T1-135, as well as 17KT, which only differs from N136 in its four C-terminal amino acids, both alter the phosphorylation status of p130; 17KT also promotes the release of E2F (Boyapati *et al*, 2003; Lin and DeCaprio, 2003). We observed an interaction between Hsc70 and JCV TAg, but were unable to convincingly demonstrate stable binding of the three T′ proteins to this cellular chaperone (data not shown). Given the findings reported for SV40 truncated T proteins, we speculate that JCV T′ proteins interact transiently with Hsc70. This expectation is supported by recent findings that demonstrate T′ proteins differentially effect the release of E2F family members from their Rb binding partners (Tyagarajan and Frisque, in preparation).

Many of TAg's functions are regulated by the modification of specific N- and C-terminal amino acid residues. The T′ proteins share the TAg phosphorylation sites at their amino terminus, and, in addition, possess potential phosphorylation sites at their shared (T'_{165}) or unique (T'_{135}) C-termini. T′ proteins migrate as doublets on polyacrylamide gels, and treatment with phosphatase resolves each protein into one, faster migrating species, suggesting that both bands of the doublet represent differentially phosphorylated forms of T′. Interestingly, the patterns of phosphorylation in dividing versus growth-arrested cells differs for each T′ protein, suggesting that the T′ proteins may be modified by different cellular kinases and phosphatases, and that some of these modifications may be cell cycle dependent. This suggestion is supported by recent work in which we mutated a cyclin-dependent kinase consensus sequence at amino acid position 125 (threonine to alanine or glutamic acid) in the JCV early coding region (Tyagarajan and Frisque, 2006). Although the mutant TAg was highly unstable and therefore not detected on our gels, the mutant T′ proteins were stable and exhibited migration patterns on gels that resembled those seen in the quiescent cells from the current study (i.e., one hypophosphorylated band). The cell-cycle dependent phosphorylation of SV40 TAg influences many of its properties, including transformation efficiency (McVey *et al*, 1989; Scheidtmann *et al*, 1991; Schneider and Fanning, 1988), and similar modifications of the JCV TAg and T′ proteins may contribute to this virus' oncogenic behavior as well (Swenson and Frisque, 1995; Tavis *et al*, 1994). One mechanism by which phosphorylation might increase transforming efficiency is through the generation of modified T′ species that exhibit enhanced binding to the Rb proteins. However, this hypothesis is not supported by our co-IP/WB analysis of the REF lines that revealed similar binding activities of both the hyperphosphorylated and hypophosphorylated forms of the T′ proteins.

In summary, we have created cell lines expressing individual JCV tumor proteins to facilitate a functional analysis of three TAg splice variants. The N-terminal transformation domains of the T′ proteins mediate interactions with p107 and p130. T'_{135} binds most efficiently to p107 and p130 and has the greatest impact on saturation density and AIG of Rat2 cells. Interaction between p107 and T'_{136} are difficult to demonstrate in G418-selected Rat2 cells, but not in REF cells selected on the basis of a transformation phenotype and expressing high levels of this JCV tumor protein. Interestingly, T'_{136}, the predominant T′ protein in extracts of JCV-transformed cells, has the most profound effect on p107 and p130 phosphorylation status. Both hypophosphorylated and hyperphosphorylated species of each T′ protein are produced in rapidly dividing rodent cells, and both forms bind p107 and p130, although their relative efficiency of binding has not been rigorously measured. TAg clearly binds to p107 and p130 present in transformed REF; TAg's inefficient interaction with these cellular proteins in R2-ΔT′ cells likely reflects poor expression of the viral protein. The basis for this low level of production and for our difficulty in deriving TAg-only expressing cells is currently under investigation. The independent expression, modification, and regulation of the JCV early proteins may provide a means for fine-tuning the activities attributed to the N-terminal region of TAg. Each of these unique interactions contributes to the overall phenotype observed in cells expressing the intact viral early region. Investigations into the consequences of the interactions between JCV early proteins and critical cellular factors, the roles of TAg and the T′ proteins in cell survival and the contributions of T′ proteins to TAg functions are continuing.

Materials and methods

DNA plasmids

The DNA constructs used to obtain JCV TAg and T′ protein expressing cell lines contain the cDNA for TAg, T'_{135}, T'_{136}, or T'_{165} under the control of the CMV promoter in the pCR3 vector (Invitrogen Life Technologies, Carlsbad, CA; pCMV-JCT, pCMV-T'_{135}, pCMV-T'_{136}, and pCMV-T'_{165}) (Bollag *et al*, 2000). The pCMV-JCV_E plasmid, which contains the intact viral early region, was constructed by replacing the intron-spanning region (nt 4413 to 4877) from pCMV-JCT with that from pM1TCR1A (Frisque, 1983) by *Pfl*MI-*Eco*NI digestion. The pCMV-ΔT′ construct, which expresses TAg only, was made by exchanging the *Pfl*MI-*Bgl*II fragment from the pCMV-JCT plasmid (nt 4242 to 4413) with the corresponding fragment from the donor splice site mutant JCVΔT′ (Trowbridge and Frisque, 1995). The pCMV-JCV_E and pCMV-ΔT′ constructs

were verified by sequencing at the Nucleic Acid Facility of the Huck Institutes of the Life Sciences at Penn State University. The J domain mutant, H42Q, and the L×C×E domain mutant, E109K, were generated from pCMV-JCV_E using a PCR-based site-directed mutagenesis approach and the primers: E109Kfwd, 5′-CTGGCAAACATTTCTT**T**ATGGCAAAACAGGTC-3′; E109Krev, 5′-GACCTGTTTT GCCAT **A**AAGAAATGTTTGCCAG-3′; H42Qfwd, 5′-CCCCACCTTTATCAGG**T**TGGAGTTCTTTGC-3′; H42Qrev, 5′-GCAAAGAACTCCA**A**CCTGATAAAGGTGGGG-3′. Once a mutation was confirmed by sequencing, an appropriate restriction enzyme fragment was isolated from the mutated vector and exchanged with the corresponding fragment from pCMV-JCV_E. This approach ensured that any secondary mutations inadvertently introduced during the PCR amplification step would not be present in the final construct. A *Pfl*MI-*Bgl*II fragment or an *Eco*NI-*Bgl*II fragment was subcloned into the pCMV-JCV_E backbone to yield the E109K or H42Q mutant, respectively.

The coding region for each JCV protein was excised from the respective CMV plasmid with *Eco*RI and inserted into the pEGFP-C3 vector (BD Biosciences, Palo Alto, CA) at its unique *Eco*RI site to create the EGFP-fusion constructs, EGFP/ΔT′, EGFP/T'_{135}, EGFP/$T'_{136,}$ and EGFP/T'_{165}. Fusion constructs were verified by restriction enzyme digestion, first with *Eco*RI and then with *Bam*HI. The small t-EGFP fusion construct (pEGFP-C2.ST3) was graciously provided by Dr. Ana Muñoz-Mármol (Hospital Universitari Germans Trias i Pujol, Barcelona, Spain).

The pPVU0 construct contains the SV40 regulatory and TAg, tAg, and 17KT protein coding regions (Kalderon and Smith, 1984). The vector 17KT intron produces the SV40 17KT protein only (Boyapati *et al*, 2003) and the vector Sp72-ras contains the coding information for the H-ras oncogene (Goldfarb *et al*, 1982; Cavender *et al*, 1995).

Cells and cell lines

Primary Fischer 344 REF were derived as described previously (Cavender *et al*, 1995) and propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml kanamycin, 0.03% L-glutamine, and 0.075% $NaHCO_3$, and maintained at 37°C in a humidified atmosphere containing 5% CO_2. The Rat2 fibroblast line was propagated similarly, except kanamycin was omitted from the medium, FBS was reduced to 5%, and the cells were incubated in 10% CO_2

G418-selected cell lines expressing early viral proteins

TAg and T′ expressing cell lines were created by seeding 60-mm plates with 4×10^5 Rat2 cells and cotransfecting the cells 16 h later with 1 μg pSV2-neo and either 5 μg of JCV DNA or 10 μg of calf thymus (CT) DNA using a modified calcium phosphate procedure (Trowbridge and Frisque, 1993). At 24 h post transfection (p.t.), the medium on the cells was changed to DMEM supplemented with 5% FBS. At 40 h p.t. cells from each 60-mm plate were trypsinized and replated onto three 35-mm plates. After 12 h the medium was replaced with growth medium containing 400 μg/ml G418. $G418^r$ cell populations were evident after about 14 days. They were maintained in 200 μg/ml G418-containing growth medium, and cell lines were cloned from single cells. Not more than 1 cloned cell line was established from each 35 mm plate of cells.

Ras cooperation assay

The Ras cooperation assay was performed in primary REF as previously described (Cavender *et al*, 1995; Beachy *et al*, 2002). Dense foci began forming between 10 and 15 days p.t. on the plates cotransfected with Sp72-ras and JCV- or SV40-expressing vectors. One to three days after their appearance, foci were either stained with a crystal violet solution and counted or removed with a Pipetman tip and transferred to 35-mm plates. These latter cells were grown to confluence and passaged at ratios of 1:100 to determine if they had become immortalized. Cells were stained with crystal violet and photomicrographs were taken at several magnifications on a Nikon Diaphot-TMD microscope and a Sony digital camera model DKC-ST5 to record cell morphologies.

Growth rates, saturation densities

The rate of proliferation of cloned cells from individual $G418^r$ lines was determined by seeding 60-mm plates with 1×10^4 cells. Culture medium containing 10% FBS was changed every 4 to 5 days and cells in duplicate plates were trypsinized and counted every 2 days. Doubling times were calculated at different time intervals using the formula $dt = t \times \ln 2/\ln(c_{t2}/c_{t1})$, where t = time (h) between cell counts, c_{t1} and c_{t2} = counts at the first and second time points, respectively. Saturation densities were measured by plating 1×10^4 cells onto 60-mm plates in medium supplemented with 1% FBS, replacing that medium every 4 to 5 days and counting cells on duplicate plates at day 35.

Growth in soft agarose

G418-selected Rat 2 cells (1×10^5) expressing either one or all five of the JCV early proteins as well as negative (R2-CT) and positive (R2-M1) control lines, were suspended in DMEM containing 10% FBS and 0.33% low melting sea plaque agarose (FMC Bio Products, Rockland, ME) and seeded onto a minimum of three 60-mm plates having a bottom layer of 0.5% agarose. Fresh suspension medium was added after 7 and 14 days, and at day 21, colonies with diameters exceeding 0.05 mm were counted in 30 randomly selected fields (1.96 mm^2/field) per plate.

DNA sequencing
The nucleotide sequence of an integrated ΔT′ cDNA in a single TAg-expressing clone of Rat2 cells (R2-ΔT′) was determined by PCR amplification and automated sequence analysis. Genomic DNA was extracted from the cells using the QIAamp Blood Kit (Qiagen, Valencia, CA). PCR was performed in an MJ Research PTC-200 Peltier Thermal Cycler using *Pfu* Turbo polymerase (Stratagene, La Jolla, CA) and the early region primers RT-PCR P5 (Bollag *et al*, 2000) and T′primer 1 (Trowbridge and Frisque, 1995). The resulting amplicon was ligated into the pCR-ScriptAmp vector (Stratagene) according to the manufacturer's protocol. The ScriptAmp-ΔT′ plasmid was sequenced by Penn State's Nucleic Acid Facility. Automated fluorescence sequencing was performed in sequential steps using various sets of primers to obtain the complete sequence of the TAg coding region.

Cellular extracts
Cells were grown in 100-mm plates to 95% confluence, washed twice with cold STE (150 mM NaCl, 20 mM Tris, pH 7.2, 1 mM EDTA), and incubated with agitation for 20 min at 4°C in 1 ml EBC buffer (50 mM Tris, pH 8.0, 120 mM NaCl, and 0.5% NP-40) containing protease inhibitors (10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml E-64, 1 mM pefabloc; Roche Diagnostics; Indianapolis, IN) and phosphatase inhibitors (5 mM NaF, 1 mM Na_3VO_4, 5 mM EDTA, and 25 mM β-glycerophosphate; Sigma, St. Louis, MO). Cell lysates were clarified by centrifugation at 16,000 × g for 20 min at 4°C. Protein concentrations of the supernatants were determined (BioRad, Hercules, CA) and the extracts stored at −80°C.

Antibodies
Monoclonal antibodies PAb 901, 962 (Tevethia *et al*, 1992), PAb 2000, 2001, 2003, 2023, 2024, 2030 (Bollag *et al*, 2000) were used to detect the five JCV early proteins. Anti-p107 (C-18) and anti-p130 (C-20) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary anti-mouse and anti-rabbit immunoglobulin G (IgG)-alkaline phosphatase (AP) conjugates were purchased from Sigma.

Immunoprecipitations and Western blots
JCV early proteins, and their binding to p107 or p130, were detected by immunoprecipitation (IP) and Western blot (WB) analysis as previously described (Bollag *et al*, 2000). Briefly, cellular extracts of Rat2 cells expressing JCV early proteins were incubated with agitation with PAb 962 monoclonal or rabbit anti-p107 or anti-p130 polyclonal antibodies for 60 min at 4°C. Antigen-antibody complexes were bound by Pansorbin cells (Staph A; Calbiochem, La Jolla, CA), suspended in EBC buffer containing 2 μg/μl bovine serum albumin (BSA) and protease inhibitors (see under extracts) for 30 min at 4°C while being agitated. The following steps were performed on ice when possible: immune complexes bound to Staph A were collected by centrifugation, washed once with 1 ml High Salt Buffer (500 mM NaCl, 50 mM Tris, pH 8.0, and 1% NP-40) and twice with 1 ml EBC buffer, both buffers containing protease inhibitors. The pellets were suspended in Sample Buffer (62 mM Tris, 10% glycerol, 2% sodium dodecyl sulfate [SDS], 5% β-mercaptoethanol, bromophenol blue, pH adjusted to 6.75), and heated at 95°C for 4 min. After centrifugation, proteins in the supernatants were separated on SDS-6%, −8.75%, or −15% polyacrylamide gels by electrophoresis in Tris-glycine running buffer (25 mM Tris, 193 mM glycine, 0.1% SDS). Proteins were transferred from gels to nitrocellulose membranes (0.2 μm pore size; Schleicher & Schuell, Keene, NH) in Hoeffer's Transfer Buffer (25 mM Tris, 193 mM glycine, 20% methanol, 0.01% SDS) overnight at 30 V in a Mini-Trans Blot Cell transfer apparatus (BioRad, Hercules, CA). The membranes were incubated twice for 30 min at room temperature (RT) in Blocking Solution (13% skim milk powder and 0.1% Tween 20 in Tris-buffered saline [TBS; 20 mM Tris, 137 mM NaCl; pH adjusted to pH 7.6]). To detect JCV early proteins the membranes were incubated with a mixture of PAb 962, 2001, and 2023, and for the detection of p107 and p130, membranes were incubated with goat anti-p107 or anti-p130, respectively, for 1 to 2 h at RT. The membranes were then washed with TBST (0.1% Tween 20 in TBS buffer) six times for a total of 15 to 20 min at RT and incubated for 1 to 2 h at RT with alkaline phosphatase (AP)-conjugated anti-mouse IgG to detect JCV proteins or anti-goat or anti-rabbit IgG to detect cellular Rb proteins. After washing the membranes again, the protein bands were visualized by developing in substrate solution buffer (100 mM Tris, 100 mM NaCl, and 50 mM $MgCl_2 \cdot 6\ H_2O$; pH 9.5) containing nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3′-indolyl phosphate p-toluidine salt (BCIP) (Life Technologies, Gaithersburg, MD).

To examine interactions between JCV tumor proteins and Rb proteins in extracts of REF, a similar co-IP/WB protocol was followed, except that a mixture of anti-T antibodies rather than anti-Rb antibodies was used in the WB step, thereby revealing all JCV proteins in a cellular extract capable of binding p107 or p130.

Nuclear localization of T′ proteins
Rat2 cells were transiently transfected with EGFP-fusion constructs using Lipofectamine 2000 reagent according to the manufacturer's protocol (Invitrogen). Briefly, 2.5 x 10^5 cells were plated in duplicate wells of a 6-well plate containing 12-mm glass coverslips. The next day, when cells had reached 90% to 95% confluence, 0.5 ml of a transfection solution containing 4 μg EGFP-fusion plasmid DNA and 10 μl Lipofectamine 2000, was added to each well and the plates were returned to the incubator. After 48 h, the coverslips were removed, rinsed in PBS, and visualized with an Olympus BX-60 epi-fluorescent microscope at Penn State's Center for Quantitative Cell

Analysis. Photographs were taken with a Hamamatsu Orca-100 digital camera, and images were colorized using ImagePro Plus software.

T′ phosphorylation in growing versus quiescent cells

Phosphorylation of T′ proteins was confirmed by treating immune complexes with λ-phosphatase (λ-PPase). Extracts from Rat2 cells expressing T'_{135}, T'_{136}, and T'_{165} were immunoprecipitated according to the protocol outlined above. Immune pellets were washed in EBC buffer, then incubated in phosphatase buffer with or without the addition of 380 U λ-PPase (New England BioLabs, Beverly, MA) according to the manufacturer's protocol. Pellets were washed in EBC buffer containing leupeptin and phosphatase inhibitors. Proteins were separated on a 20% SDS-polyacrylamide gel and detected by WB.

The phosphorylation patterns of T'_{135}, T'_{136}, and T'_{165} expressed in cloned Rat2 cell lines were compared between cells that were vigorously growing and cells that were held at confluence for 1 or 2 days (quiescent). Fifteen 100-mm plates of each cell line were cultivated in DMEM supplemented with 5% FBS. After reaching confluence, 20% of the cells were subdivided 1 to 5 and grown for 24 h in DMEM containing 10% FBS. The remaining cells were washed with, then cultivated in, DMEM containing 0.5% FBS for 24 or 48 h. After the 24-h incubation period, the vigorously growing cells and the 1-day quiescent cells were extracted with EBC lysis buffer. The extraction procedure was repeated 24 h later for the 2-day quiescent cells, and extracts were stored at −80°C. Protein concentrations were determined by the BioRad protein assay, and IP was performed on extracts of Rat2 cells expressing T'_{135}, T'_{136}, and T'_{165}. Immune complexes were separated on a 20% SDS-polyacrylamide gel and WB analysis was performed following the protocol outlined above.

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