JCV EARLY PROTEINS REGULATE RB-E2F COMPLEXES AND PROMOTE PROGRESSION OF CELLS INTO S PHASE

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by

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ABSTRACT

JC virus (JCV), a human virus closely related to Simian virus 40 (SV40), infects ~80% of the world population. In certain immunocompromised individuals an asymptomatic infection may lead to progressive multifocal leukoencephalopathy (PML), a demyelinating disease to which ~5% of AIDS patients succumb. As a member of the Polyomaviridae family, JCV readily causes tumors in experimental lab animals; recent reports associate JCV with certain human cancers. The 5 JCV early proteins TAg, tAg, T'_{135}, T'_{136} and T'_{165} contribute to transformation of non-permissive cells, and to viral replication in permissive cell types. These 2 activities are linked to the ability of DNA tumor viruses to deregulate cell cycle progression. The tumor proteins of this group of viruses override normal G_0/G_1 to S phase transition by binding to members of the retinoblastoma (RB) family of tumor suppressors through their LXCXE domain, and then causing the release of members of the E2F family of transcription factors via the co-chaperone function of their J domain. TAg and T' proteins share their N-terminal 132 amino acids sequence, which includes these 2 domains. The work herein characterized the role of JCV TAg and T' proteins in disrupting RB-E2F complexes and causing the release of E2F-1 and E2F-4, subsequently leading to the progression of cells into S phase of the cell cycle. By constructing appropriate mutants, we demonstrate that the LXCXE and J domains present in JCV TAg and T' proteins are required for interactions with the RB family members and the release of both E2F proteins. All 4 viral proteins bind to the hypo-phosphorylated forms of p107 and p130, while causing dephosphorylation and/or degradation of the hyper-phosphorylated forms of these two proteins. Further, this study
also identifies the threonine 125 residue in TAg and T' proteins as being critical for TAg stability, interaction with RB family members, induction of cell cycle progression, anchorage independent growth and viral replication. In summary, these studies contribute to our understanding of the molecular mechanisms through which JCV TAg and T' proteins promote cellular transformation and/or viral replication in cells.
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ABBREVIATIONS:

ADA, Ada(Ahx)₃-(Leu)₃-Vinyl Sulphone  
ATP, Adenosine triphosphate  
AIG, Anchorage-independent growth  
BCS, Bovine calf serum  
βgal, βgalactosidase  
CMV, Cytomegalovirus  
dCTP, 2'-deoxycytidine 5'-triphosphate  
DMEM, Dulbecco’s modified Eagle’s medium  
EGFP, Enhanced Green fluorescent protein  
FACS, Fluorescence activated cell sorting  
FBS, Fetal bovine serum  
HAT, Histone acetyl transferase  
HDAC, Histone deacetylase  
HEK, Human embryonic kidney  
HMEC, Human mammary epithelial cells  
HR, Host range  
MEF, Mouse embryo fibroblasts  
MLC, Myosin light chain  
NaF, Sodium fluoride  
NaVO₄, Sodium vandanate  
NBT/BCIP, Nitro-Blue Tetrazolium Chloride/ 5-Bromo-4-Chloro-3’-Indolyphosphate p- Toluidine Salt  
NLS, Nuclear localization signal  
PBS, Phosphate buffer saline  
PHFG, Primary human fetal glial  
PML, Progressive multifocal leukoencephalopathy  
PMSF, Phenylmethylsulfonylfluoride  
PP2A, Protein phosphatase 2A  
mPyV, Mouse polyoma virus  
RT-PCR, Reverse transcription polymerase chain reaction  
SV40, Simian virus 40  
SUMO, Small ubiquitin like modifiers  
TBP, TATA binding protein
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Chapter 1

INTRODUCTION
Members of the *Polyomavirinae* family are small, non-enveloped viruses with icosahedral capsids and circular, double-stranded DNA genomes. The first of these viruses to be identified was mouse Polyoma Virus (mPyV), which along with Simian Virus 40 (SV40), have been intensely studied since their discovery 40 years ago. These studies have shed light on a variety of eukaryotic cell processes, including DNA replication, transcription, translation, mRNA processing signal transduction and oncogenic transformation. Two polyomaviruses, JC virus (JCV) and BK virus (BKV), that solely infect humans were later identified and isolated. JCV was isolated from brain tissue derived from a patient with progressive multifocal leukoencephalopathy (PML), and BKV from the urine of a renal transplant recipient (35, 77). JC and BK are initials of the two patients from whom the viruses were first isolated. Serological surveys have shown that BKV and JCV are distributed worldwide. Each infection is thought to occur at an early age leading to lifelong asymptomatic infection in healthy individuals. In certain immunocompromised individuals, the viruses can be reactivated to cause the fatal disease PML in the case of JCV and nephropathy and kidney loss in the case of BKV. Once rare, PML is now known to be the cause of death for nearly 5% of AIDS patients (5).

In addition to being opportunistic pathogens, JCV and BKV have been associated with many human tumors. SV40 has also received much attention in recent years because SV40 sequences have been identified in several human cancer tissues (95) and references therein). Between the years 1955-1963, inactivated Salk polio vaccine prepared in African green monkey kidney cells was administered to nearly 100 million U.S. residents. Later, it was discovered that the primary simian cultures used in vaccine
production were contaminated with live SV40. During the past 10 years, sensitive PCR-based detection methods have been used to identify SV40, as well as JCV and BKV DNA sequences in variety of human tumor tissues. However, it is unknown whether polyomaviruses induced these tumors. To enhance our understanding of JCV’s oncogenic potential, experiments described in Chapters 2 and 3 were performed to examine the interactions of JCV tumor proteins with cellular tumor suppressor proteins.

1.1 Viral Genome.

The JCV genome is a closed, circular double-stranded DNA molecule- 5130 bp in the case of JCV (Mad1) strain (31), Figure 1.1). The organization of polyomavirus genome is conserved. The early genes are transcribed from one strand of the genome, and the late genes are transcribed in the opposite direction from the complementary strand. The early and late promoters, along with the viral replication origin are clustered together within the regulatory region. The JCV early precursor mRNA is alternative spliced to yield five transcripts encoding large T antigen (Tag), small t antigen (tAg), T'_165, T'_136, or T'_135. The five early proteins share their amino-terminal (N-terminal) 81 amino acids, and TAg and the three T' proteins also share the contiguous 51 amino acids. T'_165 shares its C-terminal 33 amino acids with TAg, while T'_135 and T'_136 have unique 3 and 4 amino acid C-termini respectively. The late viral mRNA encodes three capsid proteins, VP1, VP2 VP3, and a regulatory protein called LP1 or Agnoprotein.
The JCV regulatory region contains numerous, overlapping cis-acting sequences, including the early and late promoters, the enhancer and the core and auxiliary origins of DNA replication. Following early gene expression in a permissive cell, TAg binds specifically to a pentanucleotide motif within the origin sequences to initiate viral DNA replication. Once replication occurs, late gene expression ensues, leading to the production of LP1 and the three capsid proteins, VP1, VP2, and VP3. The SV40 LP1 influences the assembly of progeny virions (85), while the JCV LP1 appears to contribute

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Figure 1.1: JCV genome.
The circle in the center represents the double stranded DNA genome of JCV. The arcs to the right represent the 5 early proteins encoded by the virus, while the arcs to the left represent the late proteins. The dotted lines between the arcs represent intron regions in the early transcript. Between the two coding regions lies the regulatory region (RR).
to viral gene transcription, DNA replication, cell cycle progression (G₂ to M phase) and DNA repair mechanisms (20, 89).

1.2 Virus-Host Cell Interactions.

Polyomaviruses are non-enveloped viruses with a diameter ranging from 40 to 45 nm that encompasses the viral genome. The icosahedron capsid is composed of 72 pentamers of three structural proteins VP1, VP2 and VP3. Each VP1 pentamer is associated with a single molecule of either VP2 or VP3 (58, 82). Virus can bind to and enter cells of multiple species. In permissive cells the virus undergoes a lytic infection, while in non-permissive cells (e.g., rodent fibroblasts in the case of the primate polyomaviruses), viral DNA replication and late protein expression are blocked, resulting in an abortive infection that may lead to an oncogenic state (30). While the polyomaviruses can bind to and enter many cell types, these viruses exhibit a tropism that is mainly a function of transcriptional regulation. Cells permissive for JCV include human glial cells, lymphocytes, tubular epithelial kidney cells and tonsillar stromal cells. In vivo, JCV persists in the urinary tract and possibly brain and bone marrow of healthy hosts.

Infection of cells is initiated by binding of the virion to specific cell surface receptors. The receptor binding determinants are located on each of the VP1 monomer (106). Different polyomaviruses exploit different cellular receptors and endocytic pathways to enter cells. SV40 uses caveolae-dependent endocytosis, JCV uses clathrin-dependent endocytosis and mPyV uses a unique pathway involving uncoated pits.
Interaction between SV40 and host cells involves a direct contact between VP1 and MHC class I proteins (3). BKV interacts with a cell surface N-linked protein containing alpha (2,3)-linked sialic acid to gain cell entry (Dugan and Atwood, unpublished data). JCV has been shown to recognize two receptors, a ubiquitous N-linked glycoprotein containing α(2-6)-linked sialic acid (60), and the serotonergic receptor 5HT2AR found on a smaller number of cell types (27). It is speculated that the α(2-6)-linked sialic acid receptor found on many cell types provides initial docking for the virus and then the serotonergic receptor 5HT2AR is used to gain entry of permissive glial cells. Once the viral genome reaches the nucleus, the viral early genes are transcribed and translated and the viral DNA is replicated using cellular machinery.

1.3 Lytic and Oncogenic Potential of JCV in vivo.

Currently there is no animal model to study a productive JCV infection. Inoculation of JCV into some animals results in tumor production. Oncogenic studies have been conducted using transgenic mice expressing full-length TAg or the intact viral early region. In one such study mice developed adrenal neuroblastomas (23). Less frequently, abnormal formation of myelin sheaths in the CNS was observed in the mice leading to a shaking disorder that resembles that of myelin-deficient strains of quaking or jumpy mice (102, 103). Although some similarities to human PML were noted, this pathology does not involve cytolytic destruction of oligodendrocytes, but rather a down-regulation of myelin-specific genes by the JCV TAg (38, 122). In another transgenic study, TAg
expression under the control of its own promoter led to pituitary gland tumors (37). JCV also causes a wide variety of brain tumors including medulloblastomas and astrocytomas when inoculated interacerebrally into Syrian hamsters (128, 139). Importantly, JCV is the only human virus known to induce solid tumors in nonhuman primates. Owl and squirrel monkeys inoculated with JCV intracerebrally or intravenously develop astrocytomas, glioblastomas and neuroblastomas in 16 to 24 months (62).

In humans JCV has been detected by PCR in brain tumors, either in association with or in the absence of PML. Using primers that recognize sequences encoding the N-terminus of TAg, JCV-specific products were amplified from oligodendrogliomas, astrocytomas, pilocytic astrocytomas, oligoastrocytomas, anaplastic oligodendrogliomas, anaplastic tumors, glioblastoma multiforme and ependymomas. Furthermore, immunohistochemical staining of tissue samples confirmed the expression of TAg in several of the tumor specimens (23).

JCV has also been associated with non-neural tumors involving the gastrointestinal tract and prostrate. Although TAg expression has been detected in many JCV-associated tumors, expression of capsid proteins is not observed. These in vivo results support in vitro studies that indicate early gene expression, but not viral replication and late gene expression occurs during the oncogenic process.

1.4 Lytic and Oncogenic Potential of JCV in vitro.

JCV, unlike SV40 and BKV, exhibits a highly restricted tropism in cultured cells. JCV replicates efficiently only in primary human fetal glial (PHFG) cells and in transformed
cells derived from this heterogeneous primary culture (e.g. POJ, PHFG cells transformed with Ori defective JCV, (67); SVG, PHFG cells transformed with ori defective SV40, (66). Much effort has been expended in attempts to identify a more convenient cell system (reviewed by (49). Inefficient propagation of JCV has been demonstrated in human tonsillar stromal cells, astrocytes, Schwann cells, B lymphocytes, embryonic kidney cells and immortalized brain cell lines.

A productive JCV infection requires expression of both early and late proteins. In contrast, viral transformation of cells requires only early gene expression. SV40, and to a lesser extent BKV, induce oncogenic transformation in cells derived from a variety of tissues and host species. Given the high oncogenic potential of JCV in vivo, initial findings that the virus inefficiently transforms only a small number of cell types in vitro (mostly rodent fibroblasts and brain cells) was unexpected. Studies by our group have demonstrated that JCV’s poor transforming activity is related to both the reduced expression and activity of the viral tumor proteins (8, 123).

1.5 JCV TAg Functions.

JCV TAg is a multifunctional protein that regulates the viral life cycle through the binding of the viral replication origin sequences and a variety of cellular proteins (Figure 1.2).
Polyomavirus TAgS activate a large number of cellular and viral promoters in vitro and in vivo via interactions with a number of cellular transcription factors (86, 117). Regulation of early and late viral promoters and some cellular gene promoters occurs both in the presence and absence of direct DNA binding by TAg. Interaction of JCV TAg with Purα attenuates Purα-induced early gene transcription, decreases TAg-activated transcription of viral late promoter (17). Interaction of TAg with Tst-1 facilitates synergistic activation of both early and late promoters (84).

Figure 1.2: JCV TAg and its predicted domains.
Domains identified in JCV TAg are based in part on comparisons with the closely related SV40 TAg. The J, LXCXE and bipartite p53 binding domains are functional in JCV and are thought to be necessary for efficient transformation of cells. N-terminal sequences are predicted to be essential for efficient transactivation of cellular and viral genes. A cluster of serine and threonine phosphorylation sites (P) are found at the N- and C- termini; the N-terminal Thr125 residue is critical for viral viability. Many additional domains, including helicase, Polα binding, DNA binding, Zn finger, ATP binding and ATPase, contribute to TAg’s primary function, mediating viral DNA replication.
Activation of early gene expression is followed by viral DNA replication. Replication is activated upon TAg binding to four copies of a pentanucleotide sequence (BSII), TAg oligomerizes into a double hexamer structure leading to the distortion and unwinding of origin sequences and the recruitment of the cellular replication machinery (16, 90, 126). Upon transition from the initiation to elongation phase of DNA replication, TAg displays helicase and ATPase activities resulting in the appearance of two replication forks. Replication proceeds bidirectionally as DNA is threaded through the hexameric TAg structures, and two cellular polymerases (α and δ) and their associated cofactors copy the unwound template strands. The rate-limiting step in the replication process occurs at the termination stage, perhaps because steric constraints begin to interfere with replication fork movement (47).

Combinations of mutant, chimeric or naturally occurring variant replication origins have been examined to identify the basis for observed differences in JCV, BKV and SV40 DNA replication behavior. These studies reveal that JCV TAg binds to both the JCV and SV40 replication origins, but with reduced efficiency relative to its SV40 counterpart. While the BKV and SV40 TAgS interact productively with the JCV origin, the JCV TAg promotes replication only from its own origin. The inability of the JCV TAg to drive replication from the SV40 origin has been mapped to amino acids 82-411 (65) and to 3 nucleotide differences between the AT-rich regions of the two viral core origins (64). Overall, JCV TAg mediates replication, even from its own origin, less efficiently than do the BKV and SV40 proteins (64, 104).
1.6 Role of TAg in Cellular Transformation.

Small Polyomaviruses replicate in cells that are actively dividing because they rely upon the host cell’s machinery to copy their DNA and produce viral mRNAs and proteins (61). The early viral proteins are required to establish either a productive infection or an oncogenic state in the cells. TAg is required to push cultured cells out of Go and into S phase of the cell cycle (24). Depending on the cell type, this event may either cause active replication of the virus (lytic infection) in permissive cells or lead to transformation of a non-permissive cell, a process related to tumor formation in vivo. JCV TAg has been shown to mediate viral DNA replication and transformation less efficiently than SV40 TAg (39). JCV and SV40 both produce a small TAg, but they differ in the production of shorter truncated tumor proteins. JCV produces TAg isoforms called T'135, T'136 and T'165, whereas SV40 produces one such protein called 17kT.

The transforming activity of TAg has been localized to three regions, the J, LXCXE and p53 binding domains (105). Proteins that have an LXCXE domain interact with pRb, a tumor suppressor protein, and related family members p107 and p130. The RB members bind the E2F family of transcription factors, to negatively regulate progression of the cell cycle from Go/G1 to S phase (138). DNA tumor viruses have been shown to override these normal events by binding RB proteins through their LXCXE domain and then causing the release of E2F via the chaperone function of the J domain. TAg thus inactivates the “gatekeeper” function of the RB family members and promotes cellular proliferation (135).
Sheng and co-workers (96) proposed a model to explain the ability of TAg to initiate the progression of cells from G₀/G₁ into S phase (Figure 1.3). Members of the RB family of tumor suppressor proteins bind to the E2F family of transcription factors to negatively regulate cell cycle progression from G₀/G₁ to S phase (138). Under the appropriate conditions, cyclin-dependent kinases (cdks) phosphorylate RB proteins, leading to the release of the transcription factors and promotion of G₁ to S phase transition.

Figure 1.3: Model for cell cycle progression induced by SV40 TAg.

TAg binds to under-phosphorylated RB-E2F complex via its LXCXE domain and recruits Hsc70, a member of the DnaK family of chaperones, via the J domain. The J domain activates the ATPase function of Hsc70 to release E2F from the RB-E2F complex. Released E2F activates genes involved in the S phase progression (Sheng et al., 1997; Sullivan et al., 2000).
The model suggests that TAg disrupts this regulation by first binding to RB-E2F complexes through its LXCXE domain. Once bound, TAg, via its J domain, recruits the molecular chaperone, Hsc70, and activates this cellular protein’s intrinsic ATPase function. These interactions are believed to cause the release of E2F from the complex, thereby making it available to activate genes involved in S phase progression.

Extending the SV40 model for cell cycle progression, Sullivan et al. (112) demonstrated that sequences C-terminal to the J and LXCXE domains might be essential for stable interaction with Hsc70. In addition to cell cycle regulation, interaction of TAg with Hsc70 is essential for transformation, viral DNA replication, transactivation of viral and cellular promoters, and virion assembly (reviewed by 113).

1.7 TAg Interaction with Cellular p53.

The p53 gene is one of the most frequently mutated genes in human cancers. Due to its importance in regulating cell proliferation and monitoring the integrity of the DNA, p53 is also known as the “guardian of the genome”. The unscheduled proliferation of cells induced by small DNA tumor viruses may provoke host cells to undergo growth arrest or apoptosis via p53 upregulation. However, DNA tumor viruses have evolved mechanisms to counter this cellular response; polyomavirus tumor proteins bind to and inactivate p53. This latter event suppresses induction of the cyclin-dependent kinase inhibitor (CKI), p21, a key downstream p53 effector that promotes arrest of cells in the G1 phase of the cell cycle. JCV TAg was first shown to bind p53 in transformed hamster brain cells using
a co-immunoprecipitation approach (32). Compared to SV40 TAg, only a small subpopulation of JCV TAg appears to bind p53. Through this binding, p53 is stabilized and its levels increase dramatically in primate and rodent transformed tumor cells. Recently, SV40 small tAg was found to contribute to p53 stabilization by an unknown mechanism (120); a similar role for the JCV tAg has not yet been demonstrated.

Upregulation of p53 can either induce cell cycle arrest or apoptosis via an intrinsic pathway. Under physiological conditions p53 is controlled by an autoregulatory feedback mechanism, whereby p53 transactivates the \textit{mdm2} (murine double minute 2) gene. Mdm2 in turn directly inhibits the transcriptional activity of p53 and relocates p53 to the cytoplasm. In the cytoplasm Mdm2 functions as ubiquitin ligase and targets p53 for degradation via the ubiquitin pathway (reviewed by 127). The events occurring after TAg stabilization of p53 are not clearly understood at the moment. However, it has become increasingly clear that the consequences of the stabilization event are dependent on several factors that include cell type, species, cellular environment and the cell stress response.

The ability of TAg to inhibit a p53-mediated response has been studied using a TAg temperature-sensitive mutant (tsA58). Rat embryo fibroblasts (REFs) expressing a tsA58 TAg undergo apoptosis 24 hrs after shifting cells to non-permissive temperature (71). It is possible that the sudden release of p53 in the cells triggers apoptosis. In another assay, TAg in the absence of tAg could not extend the life span of primary rodent cells and instead induced apoptosis (53).

Earlier studies involving chimeric JCV-SV40-BKV genomes support the findings that JCV tumor proteins interact with the p53 and RB tumor suppressor proteins, but that
their ability to inactivate these key regulators is reduced relative to SV40 and BKV (8, 18, 39, 112). Characterization of two sets of JCV-SV40 chimeras, generated by swapping JCV TAg sequences containing the J domain (amino acids 1-81), LXCXE domain (amino acids 82-411) or most of the p53 bipartite binding region (amino acids 412-688), led to the following observations: (i) the substitution of the J domain of JCV TAg for that of SV40 TAg resulted in reduced dense focus formation (a transformation parameter), DNA replication and p130-E2F disruption (112), (ii) the presence of JCV sequences in place of SV40 sequences within any of the three exchanged regions of a chimera yielded lower transformation efficiencies (39), (iii) hybrid proteins containing most of the p53 binding domain of JCV TAg formed less stable complexes with p53 than did a TAg with an SV40 p53 binding motif (8, 39), and (iv) chimeric TAgs containing the JCV C-terminal region immortalized human fibroblasts more efficiently than did the intact SV40 protein (76). At the time many of these studies were conducted, it was not known that the exchange of sequences within the viral early coding regions would also affect the structures of JCV T' and SV40 17kT proteins.

1.8 JCV tAg Functions.

The present understanding of tAg functions is based on work conducted in SV40. Early mutagenesis studies of JCV and SV40 indicated that tAg mutants are viable (97), (67), but exhibit some defects in infectious virion production in cultured cells (121). Transforming potential of the SV40 mutants was found to be variable and depended upon the experimental conditions (32, 68, 101). TAg transforms many cell types in the absence
of other viral proteins, but co-expression of tAg is required to initiate transformation of certain human cells (6, 40, 79, 134). Further, tAg enhances TAg-mediated transformation when quiescent cells are employed or under conditions in which low levels of TAg are present.

The SV40 tAg also exhibits transregulatory activity that depends upon a functional J domain. These activities involve both the activation and repression of cell cycle regulators, including cyclins A and D1 and the CKI, p27 (79, 80, 129). In addition, tAg transregulates other cellular promoters through its binding to cellular protein phosphatase 2A (PP2A).

tAg’s contribution to transformation primarily involves its unique C-terminal sequences that interact with the cellular serine/threonine phosphatase PP2A (74, 88). PP2A is known to play critical roles in cell signaling through the dephosphorylation of specific substrates. SV40 tAg forms a complex with the AC core enzyme, in part, through a cysteine-containing motif (amino acids 97-103). This association modifies the substrate specificity and intracellular localization of PP2A (132), resulting in a multitude of responses that, suggest mechanisms by which tAg cooperates with TAg to induce transformation. Inhibition of PP2A by tAg results in the activation of the protein kinase C ζ isoform (PKC ζ). Activated PKC ζ triggers PI 3-Kinase, MAPK and SAPK pathways resulting in the upregulation of transcription factors including CREB, AP-1, Sp1 and NF-κB, and leading to cellular proliferation and survival (reviewed by 33).
1.9 Discovery of JCV T' Proteins.

Initial sequencing and cloning of the JCV genome (31) suggested the presence of only two early proteins TAg and tAg. Later, protein analysis of JCV-transformed rodent cells indicated the presence of an additional early protein of the size 17 kDa (8, 39). This band was thought to be a degradation product of TAg, hence it was named T' protein. However, pulse-chase experiments suggested that this band was an authentic early protein and RT-PCR analysis confirmed this prediction (124). Furthermore, protein expression studies in lytically-infected PHFG cells, revealed 3 T' proteins ranging in size from 16-23 kDa. Immunoprecipitation reactions using a monoclonal antibody directed against the N-terminus of TAg recognized all three T' proteins, while an antibody against the C-terminus recognized only the largest T' protein. Using lambda phosphatase treatment, each T' protein, T'\textsubscript{165}, T'\textsubscript{136} and T'\textsubscript{135}, was shown to be a phosphoprotein (81, 114, Bollag and Frisque, unpublished data).

Cloning and sequencing of T' cDNA’s revealed the removal of 2 introns during processing of the early mRNA. The splice donor and acceptor sites used to remove the first intron are shared by the TAg and the three T' transcripts, while a second common donor site and a unique acceptor site are employed to remove the second intron of the T' mRNAs (Figure 1.4).
Protein Functional Analysis.

Presence of TAg isoforms in several polyomaviruses and conservation in multiple JCV variants suggest they are functional. Further, these proteins contain 3 important TAg domains J and LXCXE and NLS. Sequence differences between TAg and these isoforms may result in different folding patterns and/or DNA protein binding properties. One could envision the TAg isoforms might complement or interfere with TAg functions or specify

Figure 1.4: JCV early transcripts produced by alternative splicing of the early precursor mRNA.

Five early transcripts are generated by alternative splicing. The TAg transcript shares its donor site (#4771) with the 3 T' transcripts and its acceptor site (#4426) with all 4 of the other early transcripts. The tAg mRNA has a unique donor site (#4494). Processing of the 3 T' mRNAs involves a second pair of splice sites, a shared donor site (#4274) and unique acceptor sites (#2918, 2777 or 2704).

1.10 T' Protein Functional Analysis.

Presence of TAg isoforms in several polyomaviruses and conservation in multiple JCV variants suggest they are functional. Further, these proteins contain 3 important TAg domains J and LXCXE and NLS. Sequence differences between TAg and these isoforms may result in different folding patterns and/or DNA protein binding properties. One could envision the TAg isoforms might complement or interfere with TAg functions or specify
new functions not associated with this major viral regulatory protein. To investigate possible activities of the T’ proteins, a genetic approach that altered the common donor splice site was initially taken to abolish production of T’ transcripts. Transfection of this mutant DNA into PHFG cells resulted in a 10 to 20 fold decrease in viral DNA replication. By mutating the unique T’ acceptor sites, additional mutants have been made that express T’ proteins either individually or in combination with one another. While loss of one or two T’ proteins had little effect on DNA replication behavior the triple acceptor site mutant was highly replication defective similar to the donor site mutant (81).

1.11 T’ Proteins and Cellular Transformation

Attempts to understand the transformation potential of individual T’ protein using a dense focus assay in Rat2 fibroblasts have been unsuccessful (Bollag et al., in preparation). However, G418-selected Rat 2 cells expressing T’135 formed colonies in soft agarose, a stringent transformation property. A third transformation assay, Ras cooperation, provides an indirect measurement of transformation potential of a viral protein. In this assay, primary cells which are normally growth-arrested by expressing an activated form of Ras, may be immortalized by co-expression of some viral tumor proteins. Both T’135 and T’136 were found to cooperate with Ras to induce immortalization immortalization in 100% of the cell lines tested. Approximately 50% of the lines expressing T’165 plus Ras were immortalized. However, only 2 out of 18 cell lines expressing TAg and Ras were immortalized. Further, most of the immortalized cells had a transformed cell like
phenotype, while the $T'_{135}$ lines exhibiting the aggressive growth phenotype (Frisque, unpublished data).

1.12 T’ Proteins and RB Interaction.

TAg isoforms have been identified in SV40 (17KT), mPyV (tiny T) and BKV (mini T) (87, 136, Bam and Frisque, unpublished data). But unlike SV40 17KT and mPyV tiny T, the JCV T’ proteins are produced at relatively high levels in transformed and productively-infected cells (Jones and Frisque, unpublished data). These TAg isoforms contain two of the three TAg transformation domains (LXCXE and J). Functional analysis show that the J domain of SV40 17KT compliments a DNAJ domain mutation in TAg to restore the transformation of primary human fibroblast (10). A recent study demonstrated differences between SV40 TAg and 17KT in their ability to activate the cyclin A promoter. SV40 TAg’s ability to activate cyclin A promoter depends on its interaction with PP2A, while 17KT utilizes its J domain to induce activation (100).

Mouse polyomavirus tiny T protein stimulates ATPase activity of Hsc70, although a direct stable interaction between the two proteins is not seen (87). The SV40 17kT protein binds to and reduces the levels of p130, promotes E2F activity, and stimulates cell-cycle progression of quiescent fibroblasts (10). Cells expressing only 17kT display a minimal transformed phenotype and the protein is underphosphorylated relative to TAg, reflecting the absence of C-terminal TAg sequences that regulate modification of the N-terminus (136).
Using an *in vitro* approach, Bollag et al. (9) investigated the possibility that T′<sub>135</sub>, T′<sub>136</sub> and T′<sub>165</sub> interact with RB family members. Each purified T′ protein was shown to interact *in vitro* with the hypophosphorylated forms of the RB proteins, and importantly each viral protein exhibited differential binding to pRb, p107 and p130. Similar studies conducted *in vivo* using Rat2 cells expressing individual JCV tumor proteins revealed binding of the T′ proteins to p107 and p130 (Bollag et al., in preparation). These experiments revealed that T′ proteins not only bind to hypophosphorylated p107 and p130, but that some hyperphosphorylated species of these cellular proteins are absent in the T′ expressing cell lines. Using POJ cell extracts, and a co-IP/WB assay, it was determined that at least one of the JCV early proteins bound Hsc70 (Bollag and Frisque, unpublished data).

### 1.13 Overview and Research Significance.

The ability of DNA tumor viruses to deregulate cell cycle progression is linked to their transforming behavior in non-permissive cells and replicating activity in permissive cells. Studies in SV40 have demonstrated the role of TAg J and LXCXE domains in regulating RB family members to release free E2F and promote cell cycle progression (135). Differences have been reported in the way polyomaviruses regulate activities of the RB family members. For example, the BKV TAg is expressed at much lower levels than SV40 TAg in transformed cells (41, 42). At these low levels BKV TAg does not detectably bind to RB family members, but still induces a semi-transformed phenotype in some cells and causes a decrease in RB family proteins. In addition, even in the absence
of detectable TAg-RB interactions, BKV TAg activates E2F by a mechanism that requires the LXCXE and J domains of TAg (42). It has been demonstrated that BKV, like JCV, produces substantial amounts of a TAg isoform called mini T (8, Bam and Frisque, unpublished data). Considering the differential interaction of JCV T’ proteins and TAg with RB family members, it is reasonable to predict that JCV and BKV utilize the TAg isoforms to regulate cellular functions. This prediction is further bolstered by differences observed in the expression profiles of JCV early proteins during a lytic infection. During a time course experiment, T’135 and T’136 mRNAs are seen at day 3 post-infection (pi), while the TAg and T’165 transcripts are not prominent until day 4 pi (Prins and Frisque, unpublished data). These findings might suggest an important role for T’135 or/and T’136 during the early stages of infection, either in preventing apoptosis or aiding viral DNA replication. Further, differences are seen in the post-translational modification of JCV T’ proteins based on whether the infected cell is dividing or quiescent (9), suggesting cell cycle dependent regulation of T’ proteins. The studies described in Chapters 2 and 3 examine the ability of T’ proteins to influence the G1/S transition in non-permissive cells. Further, contributions of the J and LXCXE domains and the threonine 125 residue in JCV TAg and T’ proteins to RB function and cell cycle progression are examined.

**Hypothesis:**

I hypothesize that the newly identified T’ proteins play a significant role in abrogating the function of the RB family of tumor suppressor proteins. This activity causes the release of E2F transcription factors from RB-E2F complexes, disrupts normal cell cycle control and drives cells from G1 into S phase. Further, I also hypothesize that mutation of residue
threonine 125 will alter the ability of TAg and T' proteins to interact with RB members and effect the release of free E2F, and that phosphorylation of threonine 125 residue influences cell cycle progression in growth arrested cells.

**Specific Aim 1:** The release of E2F-1 and E2F-4 from RB complexes in different JCV cell lines and its effect on cell cycle progression.

a. To demonstrate that interactions of T' proteins with RB family members result in the release of transcriptionally-active E2F from the RB-E2F complex.

b. To investigate the ability of JCV wild type (WT) and J domain and LXCXE domain mutant proteins to override cell cycle arrest induced by serum starvation.

**Specific Aim 2:** Importance of the threonine 125 residue to JCV TAg stability and T' protein function and its influence upon E2F-1 and E2F-4 release and cell cycle progression.

a. To demonstrate that a negative charge at amino acid position 125 in TAg and the T' proteins enhances binding to pRB family members resulting in effective release of transcriptionally-active E2F from the RB-E2F complex.

b. To investigate the ability of alanine and aspartic acid substitution mutants at position 125 in TAg and T' proteins to promote cell cycle progression in growth-arrested cells.
Chapter 2

JC VIRUS REGULATES E2F AND CELL CYCLE PROGRESSION VIA A NOVEL MECHANISM
2.1 ABSTRACT

The ability of DNA tumor viruses to transform non-permissive cells and replicate in permissive cells is linked to their ability to deregulate cell cycle progression. Polyomavirus tumor proteins override G0/G1 arrest and promote S phase transition by binding RB proteins through their LXCXE domain and causing release of E2F transcription factors via the co-chaperone function of their J domain. We have demonstrated that the TAg and three T’ proteins of JCV interact with pRb, p107 and p130 in vitro and in vivo. Here, we show that these viral proteins differ in their ability to disrupt RB-E2F-1 and RB-E2F-4 complexes, with T’165 exhibiting the highest activity for E2F-1 release and TAg having a minimal effect on both E2F-1 and E2F-4 release. The JCV tumor proteins altered the subcellular distribution of E2F-4 in both quiescent and dividing cells. Mutations to the LXCXE and J domains of TAg and the T’ proteins impacted their ability to bind RB proteins, to effect the release of E2F proteins from their RB partners, and to influence the presence of hyperphosphorylated species of p107 and p130 in cells. The LXCXE domain mutant failed to induce anchorage-independent growth in non-permissive rat fibroblasts, whereas a J domain mutant failed to replicate in permissive human cells. Our results indicate that JCV T’ proteins contribute differentially to the release of E2F from RB-E2F complexes, and suggest that JCV TAg, unlike SV40 TAg, plays only a minor role in this process.
2.2 INTRODUCTION

The human polyomavirus, JCV, produces five early proteins, TAg, tAg, and the three T' proteins (T'135, T'136 and T'165) that regulate host cell functions to facilitate viral replication (124, 81, 30). The genome of the related primate polyomavirus, SV40, encodes three early proteins, TAg, tAg and 17KT (136); the primary structure of the latter protein is similar to that of the three JCV T' proteins. Recent studies indicate 17KT stimulates cell-cycle progression of quiescent fibroblasts, and complements a TAg J domain mutant to transform human diploid fibroblast cells (10).

The JCV and SV40 TAgs exhibit extensive sequence homology, and many functional domains of the two proteins are conserved. The N-terminal 132 amino acids of JCV TAg are shared with its isoforms T'135, T'136 and T'165 and contain the J and LXCXE domains. T'135 and T'136 have unique C-termini, while the C-terminal 33 amino acids of T'165 and TAg are identical. Polyomavirus TAgs bind RB proteins (pRb, p107 and p130) via their LXCXE motif and interact with Hsc70, a member of the DNA-K family of co-chaperones, via their J domain. The latter interaction activates the ATPase activity of Hsc70 and induces conformational changes within the TAg-RB-E2F complex(s) to effect E2F release and deregulation of cellular proliferation (112, 135).

Members of the E2F family of transcription factors can be grouped according to their function and their ability to interact with individual RB members. E2F-1, E2F-2 and E2F-3 activate the promoters of genes involved in S phase progression, and are referred to as activating E2Fs. E2F-4 and E2F-5 repress the promoter activity of these genes and are known as repressor E2F's. Unlike other E2F family members, E2F-1 may also induce
cellular apoptosis (54). The activating E2F’s interact preferentially with pRb, whereas E2F-4 binds to all three RB members and E2F-5 generally associates with p130 (reviewed by 13). Studies indicate RB proteins interact with different E2F family members at different stages of the cell cycle to promote an orderly progression of the cell cycle (12 and references therein). Although the details of this regulation are still unclear, various models suggest overlapping, yet distinct, activities for these different RB-E2F complexes at various stages of the cell cycle.

We have shown that TAg and T’ proteins interact with RB members in vivo and in vitro (9; Bollag et al., unpublished data). These studies reveal that TAg and T’ proteins bind RB members with varying affinity, and that the viral proteins interact only with the hypophosphorylated forms of pRb, p107 and p130. Based upon studies conducted with SV40 TAg (96, 112), we expected JCV TAg and T’ proteins to effect the release of transcriptionally-active E2F from RB complexes and to induce progression of the cell cycle from G₀/G₁ to S phase. In this report we demonstrate that JCV TAg and T’ proteins vary in their ability to disrupt RB-E2F-1 and RB-E2F-4 complexes. T’₁₆₅ exhibited the highest activity for E2F-1 release, whereas T’₁₃₅ induced the release of E2F-4 more efficiently than did the other JCV early proteins. Unexpectedly, TAg alone was unable to effect the efficient release of either E2F-1 or E2F-4 from their RB partners. Mutations to the J (H42Q) and LXCXE (E109K) domains of the JCV tumor proteins inhibited the release of E2F-1 and E2F-4. Both the H42Q and E109K mutants were found to be replication-defective in human cells. Further, cells expressing the H42Q and E109K mutant proteins exhibited a reduced ability to grow under anchorage-independent conditions relative to cells expressing the wild type (WT) early proteins. We propose that
the coordination of the different functions of the JCV early proteins is critical to productive virus-host cell interactions.

2.3 MATERIALS AND METHODS

Construction and sources of expression vectors.

The J domain mutant, H42Q, and the LXCXE domain mutant, E109K, were generated in a vector encoding all five JCV early proteins (CMV-JCV$_E$), using a PCR-based site-directed mutagenesis approach and the following primers: E109Kfwd, 5′-CTGGCAAAACATTTCTTTATGGCAAAACAGGTC-3′; E109Krev, 5′-GACCTGTATTGCCATAAAGAAATGTTTGCCAG-3′; H42Qfwd, 5′-CCCCACCTTTATCAGGTTTGGAGTTCTTTGC-3′; H42Qrev, 5′-GCAAAAGAACTCCAACCTGATAAAGGTGGGG-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 CMV-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 PfiMI-BglII fragment or an EcoNI-BglII fragment was subcloned into the CMV-JCV$_E$ backbone to yield the E109K or H42Q mutants, respectively. Similarly a PfiMI-BglII fragment or an EcoNI-BglII fragment was isolated from the CMV-E109K and CMV-H42Q mutant vectors, respectively, and subcloned into the WT JCV(Mad1) genome to create the JCV(E109K) and JCV(H42Q) viruses.
Luciferase reporter plasmids containing either 4 copies of the E2F-1 promoter element (pE2F1-luc) or 4 copies of the E2F-4 promoter element (pE2F4-luc), and an isogenic construct lacking E2F promoter elements (∆E2F-luc) were kind gifts from Dr. J. R. Nevins (Duke University). A β-gal expression vector under the control of a CMV promoter (pCMV-βgal) was obtained from Dr. G. H. Perdew (Penn State University).

**Cell Culture.**

Rat2 cell lines stably expressing the intact SV40 (R2-SV40E) or JCV (R2-JCV_E) early regions, or individual JCV TAg (R2-TAg) and T' proteins (R2-T'135, R2-T'136, or R2-T'165) were generated previously by G418 selection (Bollag et al., unpublished data). To create cell lines stably expressing H42Q (R2-H42Q) or E109K (R2-E109K) mutant proteins, we transfected Rat2 cells with 1 µg of CMV-H42Q or CMV-E109K using a modified calcium phosphate protocol (8, 131). Transfected cells were propagated in Dulbecco’s modified Eagle’s medium (DMEM) containing 400 µg/ml geneticin sulfate and supplemented with 10% bovine calf serum (BCS), 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin. Surviving cells exhibiting G418-resistance were isolated at 10 days post-transfection (p.t.) and subcloned by limiting cell dilution.

**Transient Transfections and Lucifease Reporter Gene Assay.**

To test transcriptionally active free E2F in cells, R2CT cells (2.5x10^5) were grown in 6-well culture dishes containing DMEM supplemented with 10% BCS, 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin at 37°C in 10% CO₂. Cells
were co-transfected 10-12 hrs post plating, with 750 ng pE2F1-luc or pE2F4-luc, 500 ng pCMV-βgal, different concentrations of JCV TAg and T' DNA constructs using Lipofectamine 2000 reagent (Invitrogen). Cells were transfected for 3 hrs in serum and antibiotics free media as per manufacturer’s recommendations. The luciferase activity was assayed 48 hrs post transfection using a Turner TD-20e luminometer with a dual luciferase assay system (Promega), and luciferase readings were normalized to β-gal activity.

**Immunoprecipitation and Western Blot Analysis.**

Interactions between JCV early proteins and cellular RB proteins were demonstrated by a co-immunoprecipitation assay consisting of an immunoprecipitation (IP) step and a Western blotting (WB) step. The IP step involved adding specific anti-RB antibodies to cell lysates prepared in EBC buffer (50 mM Tris, pH 8.0, 120 mM NaCl, 0.5% NP-40) containing protease and phosphatase inhibitors (10 µg/ml leupeptin, 10 µg/ml aprotinin, 10 µg/ml E-64, 1 mM pefabloc, 5 mM sodium fluoride [NaF], 1 mM sodium vandadate [NaVO₄], 500 mM EDTA) to form immune complexes. The WB step involved electrophoresing the complexes on SDS polyacrylamide gels, transferring the separated proteins to membranes, adding either anti-RB or anti-T antibodies to the membrane and adding a second antibody to permit visualization of protein bands using chemiluminescence reagent (123).

WB analysis was employed to detect E2F-1 and E2F-4 in whole cell, cytosolic and nuclear extracts. Whole cell extracts were prepared with HEPES buffer (50 mM HEPES, pH 7.9, 250 mM KCl, 0.1 mM EDTA, 0.1% NP-40, 10% glycerol) containing
protease and phosphatase inhibitors (0.4 mM NaF, 0.4 mM NaVO₄, 1 µg/mL aprotinin, 1 µg/mL leupeptin, 0.5 mM phenylmethylsulfonyl fluoride [PMSF]). Nuclear and cytosolic extracts were prepared from both confluent and subconfluent cells. Subconfluent cells were propagated in growth medium (DMEM supplemented with 10% BCS) in 100 mm dishes until they reached 30% confluence. To induce quiescence, cells were grown to confluence in 100 mm dishes and then incubated for 48 h in DMEM containing 0.1% BCS. Both confluent and subconfluent cells were removed from dishes with trypsin and washed with phosphate buffered saline (PBS). Cells were suspended in 1 mL MENG buffer (25 mM MOPS, pH 7.28, 0.02% sodium azide, 2 mM EDTA, 10% glycerol), containing protease and phosphatase inhibitors (0.4 mM NaF, 0.4 mM NaVO₄, 1 µg/mL aprotinin, 1 µg/mL leupeptin, 0.5 mM PMSF, 10µg/mL E-64 and 1mM pefabloc). Cells were homogenized with a Dounce homogenizer (25 strokes) and the homogenate was spun at 2400 rpm for 20 min at 4°C. The supernatant was removed and saved as a crude cytosolic extract, and the pellet was washed twice with 1 mL MENG buffer and spun at 2400 rpm for 20 min at 4°C. The nuclear pellet was resuspended in MENG buffer containing 500 mM sodium chloride and incubated on ice for 1 h with occasional rocking. The crude cytosolic and nuclear fractions were spun at 13,000 rpm for 45 min at 4°C and the supernatants were collected as nuclear and cytosolic extracts. The presence of E2F-1 and E2F-4 in each of the extracts was detected by immunoprecipitating these proteins with specific anti-E2F antibodies, electrophoresing the immune complexes on SDS-polyacrylamide gels and transferring the proteins onto nitrocellulose membranes (0.2 µm PROTRAN®, Schleicher and Schuell). The membranes were blocked with TBS-T buffer (10 mM Tris-base, pH 7.6, 150 mM NaCl, 0.05% Tween-20) containing 7%
milk for 1 h by rocking at room temperature. Membranes were then incubated in 4 ml blocking solution containing the appropriate primary antibodies (20 µg of anti-E2F-1 or anti-E2F-4) for 1.5 h at room temperature. Antibodies to the nuclear TATA binding protein (TBP) or cytoplasmic myosin light chain (MLC) protein were included as well. These proteins served as loading controls as well as indicators of the efficiency of our nuclear and cytoplasmic extraction procedures. Membranes were washed in TBS-T buffer for 15 min and incubated with secondary antibody conjugated to horseradish peroxidase (diluted 1:13,000) for 1 h at room temperature. Membranes were developed using the Super Signal West Pico system (Pierce) and exposed to film. The relative distribution of E2F-1 and E2F-4 in cell lines was quantitated after normalizing to TBP or MLC levels. The exposed films were scanned and the protein band intensities quantitated using ImageQuant 5.2 software (Molecular Dynamics).

**Antibodies for Immunoprecipitation and Western Blot Analysis.**

Anti-T monoclonal antibodies PAb 962; (119), PAb 2001 and PAb 2023 (9), anti-p107 (c-18; Santa Cruz), anti-p130 (c-20; Santa Cruz), anti-E2F-1 (c-20; Santa Cruz), anti-E2F-4 (c-20; Santa Cruz), anti-MLC (FL-172; Santa Cruz), and anti-TBP (SI-1; Santa Cruz) were used as primary antibodies. Anti-rabbit IgG HRP conjugate (sc-2004; Santa Cruz) and anti-mouse IgG alkaline phosphatase conjugate (A-3562; Sigma) were used as secondary antibodies.
**DpnI Replication Assay.**

U87MG cells (4×10^5) were seeded onto 60 mm dishes and transfected the following day with full-length JCV DNA (400 ng) using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s protocol. Low molecular weight DNA was extracted from the cells at 0, 7, 10 and 14 days p.t. by the Hirt procedure (45). A portion of the DNA was cleaved with the restriction enzymes DpnI and EcoRI, and digestion products were separated by electrophoresis on a 0.8% agarose gel. DNA fragments were transferred to a nylon membrane using a Rapid Downward Transfer System and an alkaline transfer protocol (Schleicher and Schuell, Amersham). The immobilized samples were hybridized with linear JCV DNA labeled with [α-^32^P] dCTP (Oligo Labeling kit, Pharmacia). Relative replication activities of different viral DNAs were determined by quantitating band intensities using ImageQuant 5.2 software (Molecular Dynamics).

**Anchorage Independent Growth.**

Parental and G418-resistant Rat2 cells were suspended in DMEM containing 10% FBS plus 0.33% Sea plaque agarose and seeded onto 60 mm dishes coated with a layer of 0.5% agarose medium. Three dishes were seeded with cells from each line (1×10^5 cells/dish). Fresh medium was added to the plates every 7 days, and after 3 weeks, colonies with diameters exceeding 0.05 mm were counted in 20 to 30 randomly-selected fields (0.65 mm^2). The percentage of cells developing into colonies was then calculated as described (8).
Cell Cycle Analysis.

Cells (2.2×10^5) from each of our G418-selected lines were seeded onto 60 mm dishes in DMEM containing 0.01% BCS, and after 72 h, DMEM containing 10% BCS was added to each dish. Cells were removed from the dishes with trypsin at 0, 9, 12 and 15 h post-stimulation and pelleted. After washing in PBS, cells were pelleted and resuspended in 300 µl PBS. Cells were fixed in 300 µl 70% ethanol, and after 2 h the cells were pelleted, washed in PBS and pelleted once more. Cells were suspended in 500 µl PI buffer (10 ml PBS, 0.1% Triton X-100, 5 µl 2 mg/ml RNase and 200 µl 1 mg/ml propidium iodide). Samples were incubated at 37°C for 15 min and then subjected to flow cytometric analysis (XL-MCL Coulter machine, Center for Quantitative Cell Analysis, Penn State University).
2.4 RESULTS.

**Derivation of Rat2 cell lines expressing H42Q and E109K mutant proteins.**

The LXCXE and J domains of SV40 TAg are required for its interaction with RB family members and for E2F release from the RB-E2F complex (42, 135). Further, these domains are critical to TAg’s ability to overcome the growth arrest of Saos-2 [Rb-1 (-/-)] cells induced by overexpression of RB family members (135). To determine whether the J and LXCXE domains of JCV early proteins contribute to the release of functional E2F from the RB-E2F complex, mutations were introduced into the JCV early coding region. The resulting J domain mutant (H42Q) and LXCXE domain mutant (E109K) DNAs were transfected into Rat2 cells to establish stable lines. Cells expressing mutant viral proteins at levels similar to those detected in R2-JCV	extsubscript{E} cells were selected for further analysis. Viral proteins immunoprecipitated from R2-E109K cells migrated faster on SDS-polyacrylamide gels than those in extracts of R2-JCV	extsubscript{E} cells (Figure 2.7 arrowheads). Similar findings were reported for the corresponding SV40 and BK virus (BKV) mutant TAgss (42, 135). To ensure that changes in migration patterns of the E109K mutant TAg and T’ proteins were not due to altered mRNA splicing or protein phosphorylation, RT-PCR analysis and phosphatase treatment of viral proteins were performed (data not shown). These experiments confirmed that there were no differences in the sizes of WT and mutant transcripts and that new phosphorylation patterns were not responsible for altered migration of E109K proteins. Therefore, it is likely that a change in the amino acid charge contributes to the observed gel migration differences.
The ability of the mutant JCV proteins to bind p107 and p130 \textit{in vivo} was examined using extracts of the R2-H42Q and R2-E109K cell lines and an IP/WB approach (Figure 2.1). Viral protein interactions with pRb were not detected in these experiments, presumably because of the low levels of pRb in rodent cells (133). Complex formation between JCV early proteins and p107 and p130 was observed in extracts of R2-JCV\textsubscript{E} and R2-H42Q cells, but not in 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, WT JCV proteins in R2-JCV\textsubscript{E} cells interacted only with the faster migrating form of p107 and p130, while the J domain mutant proteins in R2-H42Q cells bound to both the slower and faster migrating forms of p107 and p130. The LXCXE domain mutant proteins failed to interact with, and to alter the phosphorylation state of, the two RB family members. These observations confirm that interactions of JCV TAg and T' proteins with p107 and p130 require the LXCXE domain, while the degradation and/or dephosphorylation of hyperphosphorylated p107 and p130 requires the J domain.
JCV early proteins send p130 for degradation via the 26S proteosome.

Unlike in R2-JCV<sub>E</sub> cells, the hyperphosphorylated form of p130 is stable in R2-E109K and R2-H42Q cell lines. To determine if this observation is simply due to the absence of hyperphosphorylated forms in R2-JCV<sub>E</sub>, or because WT proteins are unable to bind these forms even if present, the ability of JCV early proteins to bind
hyperphosphorylated p130 *in vivo* was examined using extracts of R2-JCV$_E$ cells in an IP/WB approach (Figure 2.2). The WT cells were treated with the 26S proteasome inhibitors Ada(Ahx)$_3$-(Leu)$_3$-vinyl sulphone (ADA) or MG-132 to see if the hyperphosphorylated p130 band is targeted for degradation in these cells. Furthermore, if the treatment inhibits the degradation of hyperphosphorylated p130, IP for JCV early proteins and Western blot for p130 will tell us if JCV proteins interact with both the slower and faster migrating forms of p130. In this assay complex formation between JCV early proteins and p130 was observed in both the untreated and proteasome inhibitor treated R2-JCV$_E$ extracts. Similar to the earlier observation (Figure 2.1), the viral proteins associated with only a fraction of the total pool of p130. Also, their interaction was with only the hypophosphorylated form of p130 even when the hyperphosphorylated form was rescued from degradation in proteosome inhibitor treated samples. The appearance of hyperphosphorylated p130 upon treatment with proteosome inhibitor suggests that the slower running form of p130 is degraded in the presence of JCV early proteins.
E2F-1 levels in cell lines expressing JCV early proteins.

T' proteins contain intact J- and LXCXE- domains and bind to members of the RB family (124); Bollag et al., manuscript in preparation); therefore, we predicted that these proteins would effect the release of members of the E2F family of transcription factors from RB-E2F complexes. Prior to testing this prediction, we examined steady state levels of E2F-1 in various cell lines using an immunoblotting assay and whole cell extracts prepared from asynchronous cell lines expressing individual T' proteins or all five early WT or mutant proteins. As reported by Harris and co-workers (42) using similar BKV
and SV40 transformants, similar levels of E2F-1 were observed in cell lines expressing JCV proteins (Figure 2.3).

E2F-1 levels fluctuate during the cell cycle, with higher levels detected during S phase and lower levels during Go phase (56). R2-SV40E cells cannot be growth arrested and have a transformed cell like phenotype, which could explain higher levels of E2F-1 detected in these cells.

Figure 2.3: Detection of E2F-1 in G418-selected Rat 2 cell lines. Whole cell extracts were prepared from R2CT, R2-JCVE, R2-SV40E, R2-T135, R2-T136, R2-T165, R2-TAg, R2-E109K and R2-H42Q cells. Total protein (10 µg) was electrophoresed on a 7.5% SDS- polyacrylamide gel and transferred to a nitrocellulose membrane. Detection of E2F-1 was achieved by WB analysis using anti-E2F-1 antibody. R2-SV40E was used for positive control and MLC protein was used as a loading control, and was detected with FL-172 antibody. Protein bands were visualized using anti-rabbit IgG alkaline phosphatase conjugate and NBT/BCIP substrate solution.
**Total E2F-1 levels in cell lines expressing JCV early proteins.**

The differences in E2F-1 levels are likely to be masked in an asynchronous cell population (Figure 2.2); therefore, we compared extracts from dividing and quiescent cells to determine the differences in the E2F-1 levels. Since E2F-1 localizes to the cell’s nuclear compartment (83 and references therein), nuclear extract was prepared from the cell lines to enrich for E2F-1 levels. Furthermore, the cells were divided into two groups based on their growth confluence to aid distinguishing differences in E2F-1 levels. E2F-1 expression in confluent (cells 100% full in 100 mm dish and serum starved with 0.01% serum for 48hrs to growth arrest the cells) (Figure 2.4A) and subconfluent (cells < 50% full in 100 mm dish in an actively dividing state) (Figure 2.4B) cells were observed using immunoblot assay. The results obtained in this study support our earlier observation using asynchronous cells that differences in the steady state levels of E2F-1 are not apparent in our G418-selected Rat2 lines.
Expression and localization of E2F-4 in cells producing JCV early proteins.

E2F-4 is the predominant E2F family member in mammalian cells, and it is detected at significant levels during each phase of the cell cycle (72). Endogenous E2F-4 is found primarily in the nucleus during Go; it begins to relocate to the cytoplasm as cells enter the cell cycle (59). E2F-4 is also redirected to the cytoplasm in the presence of SV40 TAg (111). To determine whether JCV early proteins influence the level of E2F-4,

Figure 2.4: Detection of E2F-1 in nuclear extracts of confluent and subconfluent G418-selected Rat 2 cell lines.

Nuclear extracts were prepared from confluent (A) or subconfluent (B) R2CT, R2-JCVE, R2-SV40E, R2-T'135, R2-T'136, R2-T'165, R2-TAg, R2-E109K and R2-H42Q cells. Total protein (8 µg) was electrophoresed on a 7.5% SDS polyacrylamide gel and transferred to a nitrocellulose membrane. E2F-1 was detected on the immunoblot using anti-E2F-1 antibody. TBP served as a loading control and nuclear protein marker and was detected with SI-1 antibody (arrowhead). Protein bands were visualized using anti-rabbit IgG HRP conjugate and Pico chemiluminescent substrate solution.
WB assays were conducted using whole cell extracts from unsynchronized cells. As seen with E2F-1, steady state levels of E2F-4 were similar in multiple cell lines expressing the various JCV early proteins (Figure 2.5).

To determine whether JCV tumor proteins alter the subcellular localization of E2F-4, we performed WB on nuclear and cytosolic fractions prepared from subconfluent and confluent cells. In dividing cells, the distribution of E2F-4 in the nucleus vs. the cytoplasm varied depending upon the cell line examined (Figure 2.6). Lines expressing
T′\textsubscript{165}, TAg, or JCV early proteins containing the E109K or H42Q mutations contain more nuclear E2F-4 than that found in cells expressing the intact WT JCV (JCV\textsubscript{E}) or SV40 (SV40\textsubscript{E}) early coding regions, T′\textsubscript{135}, T′\textsubscript{136} or the control G418-resistant Rat2 (R2CT) cells (Figure 2.6A). Alternatively, cytosolic levels of E2F-4 were inversely related to the levels detected in nuclear extracts (Figure 2.6B). As expected, TBP was detected only in the nuclear fraction, while MLC was confined to the cytosolic fraction (data not shown). This data indicates that the subcellular localization of E2F-4 in subconfluent cells is influenced by the presence of JCV early proteins. E2F-4 localizes to the nucleus in confluent cells.

To determine if JCV early proteins alter the localization of E2F-4 in such cells, we repeated the experiments described above using cells maintained in G\textsubscript{o}/G\textsubscript{1}. E2F-4 relocalizes to the cytoplasm in cells expressing JCV\textsubscript{E}, SV40\textsubscript{E} and T′\textsubscript{135}; nuclear levels are reduced, while cytoplasmic levels are increased, relative to the levels in R2CT, R2-T′\textsubscript{136}, R2-T′\textsubscript{165}, R2-TAg, R2-H42Q and R2-E109K cells (Figure 2.6C and 2.6D).
Differential release of transcriptionally-active E2F-1 by JCV early proteins.

JCV TAg and T' proteins are expected to effect the release of activating E2Fs in complex with RB family members. We tested this prediction by conducting luciferase reporter assays in G418-selected Rat 2 cell lines expressing the different JCV early proteins. In preliminary IP/WB experiments, variation was noted in the amounts of viral...
proteins expressed in independent cloned cell lines. Because differences in viral protein expression would likely affect reporter assay results, we employed cell lines that expressed individual JCV early proteins at levels similar to those detected in JCV_E-1 (Figure 2.7 and Table 1). In making the decision about which lines to use, we took into account the observation that the three T' proteins are not produced at equivalent levels in cells expressing the intact WT early region (JCV_E); T'_136 is expressed at higher levels than either T'_135 or T'_165. It should also be noted that after screening more than 30 G418-resistant clones, only one TAg line was isolated and the level of TAg expressed in these cells was much lower than that found in R2-JCV_E cells (Bollag et al., unpublished data).
Figure 2.7: Expression of JCV proteins in G418-selected Rat2 cell lines clone set #1 and clone set # 2.

Whole cell extracts were prepared from two independent clones each of R2-JCV, R2-SV40, R2-T'_{135}, R2-T'_{136}, R2-T'_{165}, R2-E109K and R2-H42Q cells, or from one clone each of R2CT and R2-TAg cells. Viral proteins were immunoprecipitated from 800 µg of total protein in each lysate using PAb 962 antibody, separated on 15% SDS polyacrylamide gels and transferred to nitrocellulose membranes. JCV early proteins were detected by WB analysis using a mixture of monoclonal antibodies, PAb 962, PAb 2001 and PAb 2023. Protein bands were visualized using anti-mouse IgG alkaline phosphatase conjugate and NBT/BCIP substrate solution. The central portion of the blot containing bands representing antibody light and heavy chains was omitted to show the viral proteins only. Altered migration of E109K protein bands are indicated with an arrow.
Stable Rat 2 lines expressing one or more JCV early proteins were co-transfected with E2F-1 luciferase (pE2F1-luc) and β-gal (pCMV-βgal) constructs, and the activities of these reporter genes were measured. Luciferase activity was normalized based on the β-gal measurements, and the fold activity was expressed relative to the R2CT control line. Luciferase activity in cells transfected with the promoter-less pΔE2F-Luc construct was nearly undetectable (data not shown). Greater than five-fold induction in E2F-1 activity was measured consistently in the R2-T'165 cells; approximately three-fold induction occurred in the R2-JCV_E and R2-SV40_E lines. None of the other cell lines yielded more than a 1.7 fold increase in reporter activity (Figure 2.8).

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>Proteins Expressed</th>
<th>Level of Protein Expression</th>
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<tr>
<td></td>
<td></td>
<td>Clone #1</td>
</tr>
<tr>
<td>CMV-JCV_E</td>
<td>TAg, tAg, T'135, T'136 and T'165</td>
<td>++</td>
</tr>
<tr>
<td>CMV-SV40_E</td>
<td>TAg, tAg and 17KT</td>
<td>++</td>
</tr>
<tr>
<td>CMV-T'135</td>
<td>T'135</td>
<td>+</td>
</tr>
<tr>
<td>CMV-T'136</td>
<td>T'136</td>
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<td>T'165</td>
<td>+</td>
</tr>
<tr>
<td>CMV-TAg</td>
<td>TAg</td>
<td>+</td>
</tr>
<tr>
<td>CMV-H42Q</td>
<td>TAg, tAg, T'135, T'136 and T'165</td>
<td>+</td>
</tr>
<tr>
<td>CMV-E109K</td>
<td>TAg, tAg, T'135, T'136 and T'165</td>
<td>++</td>
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</tbody>
</table>
In order to make sure that the luciferase reading obtained earlier was not clone specific, but a true representation of E2F-1 release in different JCV cell lines, I repeated this experiment using clone set #2. Differences were observed, especially for T'_{165} which had a robust E2F-1 activity in clone set #1, exhibited only basal activity in clone set #2. The other changes observed were more modest- JCV_E and SV40_E in both cases were ~3

Figure 2.8: E2F-1 induced luciferase activity in G418- selected Rat 2 cell lines expressing JCV early proteins; clone set #1.

R2CT, R2-JCV_E, R2-SV40_E, R2-T'_{135}, R2-T'_{136}, R2-T'_{165}, R2-TAg, R2-E109K and R2-H42Q cells (2.5x10^5) were seeded into 6 well (35 mm) plates. After 12 hrs cells were transfected with 750 ng pE2F1-Luc and 500 ng pCMV-βgal using Lipofectamine 2000 reagent and incubated at 37°C for 3 hrs. Cells were lysed 48 hrs later and extracts were assayed for luciferase and β-gal activities. The luciferase activity for each sample was normalized using the β-gal readings, and plotted as a fold induction relative to the activity measured in the parental R2CT cell line (value of 1.0). The experiment was repeated 4 times in duplicate. The error bars represent the range between duplicate samples in this representative experiment.

In order to make sure that the luciferase reading obtained earlier was not clone specific, but a true representation of E2F-1 release in different JCV cell lines, I repeated this experiment using clone set #2. Differences were observed, especially for T'_{165} which had a robust E2F-1 activity in clone set #1, exhibited only basal activity in clone set #2. The other changes observed were more modest- JCV_E and SV40_E in both cases were ~3
fold, whereas T′<sub>135</sub> and T′<sub>136</sub> had < 2 fold increase, and TAg, E109K and H42Q remained low (Figure 2.9).

![Graph showing luciferase activity](image)

Figure 2.9: E2F-1 induced luciferase activity in G418-selected Rat 2 cell lines expressing JCV early proteins; clone set #2.

R2CT, R2-JCVE, R2-SV40E, R2-T′<sub>135</sub>, R2-T′<sub>136</sub>, R2-T′<sub>165</sub>, R2-TAg, R2-E109K and R2-H42Q cells (2.5x10<sup>5</sup>) were seeded into 6 well (35 mm) plates, and the assay was done as described for clones #1 (Figure 2.8). The experiment was repeated 4 times in duplicate. The error bars represent the range between duplicate samples in this representative experiment.

Also, it is important to note that there is correlation between higher protein expression by a clone from either set #1 or set #2 and the fold induction measured in the luciferase assay. The difference in E2F-1 luciferase activity seen between the two R2-T′<sub>165</sub> clones suggested a requirement for a minimum threshold of protein expression to induce E2F-1 release. To test this, multiple R2-T′<sub>165</sub> cell lines were assayed for T′<sub>165</sub>
protein expression, and then assayed for E2F-1 induction using the IP/Western blot and luciferase assay (Figure 2.10). T′_{165} clones 1C.30 and 2A.1 shown in Figure 2.10 are identical to T′_{165} clone set # 1 (Figure 2.8) and clone set # 2 (Figure 2.9), respectively.

Figure 2.10: Protein expression and E2F-1-induced luciferase activity in G418-selected Rat2 cell lines expressing T′_{165} proteins.

(A) Whole cell extracts were prepared from independent clones of R2-T′_{165} cells and assayed for viral protein expression from 800 µg of total protein by IP. (B) The luciferase assay was conducted as described earlier. The error bars represent the range between duplicate samples in this representative experiment. The experiment was done twice.
Release of transcriptionally-active E2F-1 following transfection of Rat2 cells with JCV expression plasmids.

Differences in fold induction between clone set #1 and clone set #2 made it difficult to interpret the role of TAg and T' proteins in E2F-1 induction. Although, clone set # 1 and clone set # 2 are independent clones, TAg clone (R2-TAg) in both sets are the same. It has been hard to isolate clones expressing TAg, and even the single clone obtained produces low levels of TAg. Considering that reduced protein expression yields low luciferase reporter activity in cells, the inability of TAg to efficiently release transcriptionally-active E2F-1 suggested either that low TAg expression levels were responsible for this observation or that TAg had little effect upon E2F-1 release. To investigate these possibilities, an alternative approach was taken that did not rely upon the use of stable G418-resistant cell lines. Rat 2 fibroblasts were co-transfected with varying amounts of CMV-TAg, CMV-JCV E, CMV-T'135, CMV-T'136, CMV-T'165, CMV-E109K or CMV-H42Q DNA, plus the pE2F1-luc and CMV-βgal constructs (Figure 2.11). In agreement with the first assay, TAg had only a minimal effect on the induction of luciferase activity (<1.5 fold), whereas T'165 had a significant impact on activation of the reporter gene. T'135 and T'136 induced at levels similar to that observed in clone set # 2 (Figure 2.8). In both assays the levels of luciferase activity induced by H42Q mutant proteins exceeded those obtained with TAg alone or the E109K mutant proteins, suggesting that the H42Q mutant retains some J domain function.
Double mutant H42Q/D44N retains some J domain function.

Studies examining the requirements for functional LXCXE and J domains in the JCV early proteins suggested that H42Q mutant retains some of the J domain function. The J domain mutant proteins (H42Q), though defective in reducing the levels of hyperphosphorylated RB proteins p107 and p130, exhibits partial release of E2F-1. In our attempt to abolish J domain function, we introduced another mutation into the HPD motif

Figure 2.11: E2F-1-induced luciferase activity in transiently transfected R2CT cells. R2CT cells (2.5x10^5) were seeded in 6 well (35 mm) plates. After 12 h the cells were cotransfected with 100, 250, 500 or 750 ng of JCV DNA (100, 500 ng transfection data shown) along with 750 ng pE2F1-Luc and 500 ng CMV-βgal using Lipofectamine 2000 reagent. The fold induction of luciferase activity was calculated as indicated in the legend to Figure 2.7. The error bars represent the standard deviation between three independent experiments.
to create the double mutant (H42Q/D44N). J domain activity was tested in a transient co-
transfection assay. Rat 2 fibroblasts were co-transfected with varying amounts of CMV-
H42Q/D44N DNA, plus the pE2F1-luc and CMV-βgal constructs (Figure 2.12). The
double mutant H42Q/D44N exhibited reduced luciferase activity compared to the single
mutant H42Q, but this activity was still greater than that observed with the control vector.
This suggested that the H42Q/D44N double mutant still retains some J domain function.

Figure 2.12: E2F-1-induced luciferase activity in transiently transfected R2CT cells.
R2 cells (2.5x10^5) were seeded in 6 well (35 mm) plates. After 12 h the cells were co-
transfected with 100, 250, 500 or 750 ng of H42Q/D44N mutant JCV_E DNA, along with
750 ng pE2F1-Luc and 500 ng CMV-βgal using Lipofectamine 2000 reagent. The fold
induction of luciferase activity was calculated as indicated earlier. The error bars
represent the standard deviation between three independent experiments.
Differential regulation of E2F-4 release by JCV early proteins.

Differences in the nuclear-cytoplasmic distribution of E2F-4 (Figure 2.5) suggest that E2F-4 is differentially regulated by JCV early proteins. Furthermore, due to relocalization of free E2F-4 into the cytoplasm, we expected to see a reduced luciferase activity in cell lines expressing proteins that disrupted RB-E2F-4 complexes. To explore these possibilities, our collection of G418-selected Rat2 cell lines were co-transfected with pE2F4-luc and CMV-βgal, and luciferase activity was measured (Figure 2.13). Minimal reporter activity was measured in transfected R2-SV40E, R2-JCV\textsubscript{E} and R2-T\textprime\textsubscript{135} cells, indicating that E2F-4 was released from RB complexes and shuttled to the cytoplasm. Higher luciferase activities were detected in the transfected R2-TAg, R2-T\textprime\textsubscript{136}, R2-T\textprime\textsubscript{165}, R2-E109K and control R2CT cells. Luciferase readings were reduced slightly in the R2-H42Q cell line, suggesting that the mutation may not have abolished the ability of the JCV proteins to release E2F-4 from RB complexes.
As with our E2F-1 experiments, we sought independent confirmation of the E2F-4 results obtained using the stable cell lines. R2CT cells were co-transfected with varying concentrations of our JCV expression plasmids plus the pE2F4-luc and CMV-βgal

Release of E2F-4 following transfection of Rat2 cells with JCV expression plasmids.

Figure 2.13: E2F-4 repressed luciferase activity in G418-selected Rat 2 cell lines expressing JCV early proteins.

R2CT, R2-JCV_E, R2-SV40_E, R2-T'_{135}, R2-T'_{136}, R2-T'_{165}, R2-TAg, R2-E109K and R2-H42Q cells (2.5x10^5) were seeded in 6 well (35 mm) plates. After 12 h the cells were transfected with 750 ng pE2F4-Luc and 500 ng pCMV-βgal using Lipofectamine 2000 reagent. The % decrease in luciferase activity was calculated after setting normalized R2CT values at 100%. The experiment was repeated 4 times in duplicate. The error bars represent the range between duplicate samples in this representative experiment.
vectors (Figure 2.14). Results were consistent between experiments using stable cell lines and transient transfections, with TAg and T'165 having minimal or no effect on the release of E2F-4, and the intact JCV early region promoting the greatest release of this transcription factor. The ability of the T'135 construct to influence E2F-4 release was less pronounced in the transient assay (compare Figs. 2.11 and 2.12).

Figure 2.14: E2F-4 induced luciferase activity in transiently transfected R2CT cells. R2 cells (2.5x10^5) were seeded in 6 well (35 mm) plates. After 12 hrs the cells were co-transfected with 100, 250, 500 or 750 ng of JCV DNA (100, 500 ng transfection data shown) along with 750 ng pE2F4-Luc and 500 ng CMV-βgal using Lipofectamine 2000 reagent. The % decrease in luciferase activity was calculated based on normalized R2CT values, which was set at 100%. The error bars represent the standard deviation between three independent experiments.
Cell cycle distribution of Rat2 cell lines expressing JCV early proteins.

The ability of viral tumor proteins to mediate the release of transcriptionally active E2F-1 and E2F-4 from RB-E2F complexes is expected to influence a cell’s progression from G₀/G₁ into S phase. To investigate this possibility, we followed the progression of synchronized populations of cells expressing JCVₑ, T₁₃₅, T₁₃₆, T₁₆₅, TAg, E109K, H42Q and SV40ₑ through G₀/G₁, S and G₂/M phases by FACS analysis. Parental Rat 2 (R2) and G418-selected Rat 2 (R2CT) control lines were included to identify potential differences in cell cycle patterns arising from the G418-selection process. Following serum deprivation (0 h), greater than 80% of the cells from each of the lines expressing JCV proteins were in G₀/G₁; cells from the SV40 control line could not be growth-arrested even when incubated 72 h in medium supplemented with 0.01% serum (Figure 2.15). At 9 h post-serum stimulation, JCVₑ cells began entering S phase. This transition was not observed for the other JCV-expressing cell lines until the 12 h time point. Relative to the R2CT control line, a higher percentage of R2-T₁₃₆ and R2-T₁₆₅ cells (48% and 54%) and a lower percentage of R2-T₁₃₅ and R2-H42Q cells (18% and 29%) were in S phase. Most of the cells from each line had entered S phase by 15 h post-serum stimulation and had completed one round of division by the 24 h time point (data not shown).
Figure 2.15: Cell cycle analysis of G418-selected Rat2 cell lines expressing JCV early proteins.

R2CT, R2-JCV1, R2-SV40, R2-T135, R2-T136, R2-T165, R2-TAg, R2-E109K and R2-H42Q cells (2.2x10^5) were seeded in 6 well (35 mm) plates. After 3 h the medium was replaced, and cells were incubated in DMEM containing 0.01% BCS to induce growth arrest. After 72 h, cells were then refed with DMEM containing 10% BCS and analyzed for cell cycle progression at 0, 9, 12 and 15 h post-serum stimulation. The percentage of cells in G1, S and G2/M phase for each cell line at each time point was determined using an XL-MCL Coulter machine (Center for Quantitative Cell Analysis, Penn State University). Results from one of three independent experiments are shown.
J and LxCxE domain functions of JCV early proteins contribute to viral DNA replication.

Interactions between SV40 early proteins and RB members affect both viral transforming and lytic activities (1, 4). Results from our anchorage independent growth assay indicate that mutations to the J and LXCXE domains of the JCV early proteins disrupt these interactions and impact transforming activity of the virus. To investigate whether these mutations also alter JCV lytic activities, we compared the viral DNA replication potential of mutants H42Q and E109K to that of WT JCV (Mad1). Viral DNAs were transfected into U87MG cells, and DNA was collected at 0, 7, 10 and 14 days p.t. using Hirt’s extraction protocol. The purified DNA was digested with restriction enzyme DpnI in order to eliminate the input DNA during analysis. The DNA samples were electrophoresed on an agarose gel, and after transferring the samples to nitrocellulose membrane the replicated DNA was analyzed using Southern blot analysis. JCV (Mad1) DNA replication activity was detected at 7, 10 and 14 days p.t.; the H42Q and E109K mutants were replication-negative and replication-defective, respectively (Figure 2.16). The intensities of bands representing replicated DNA were diminished at day 14 p.t. reflecting the reduction in cell numbers due to viral cyto-pathic effect (CPE).
Figure 2.16: DNA replication activities of intact JCV genomes expressing WT or LXCXE and J domain mutant proteins.

U87MG cells (5x10^5) were seeded in 60 mm plates. After 12 h the cells were transfected with JCV (Mad1), E109K or H42Q viral DNA using Lipofectamine 2000 reagent. After 3 hrs medium containing 10% FBS was added to the transfected cells, and at days 0, 7, 10 and 14 p.t., samples were collected using the Hirt procedure. Replicated samples were analyzed from two independent experiments. The band representing the replicated DNA is indicated (arrowhead).
The LxCxE domain in JCV early proteins is required to induce anchorage-independent growth (AIG).

Conflicting results have been reported in previous studies regarding the contributions of the LXCXE and J domains of SV40 TAg to the establishment of anchorage-independent cell growth. Stubdal and colleagues (110) reported that only an intact LXCXE domain was required, whereas Tevethia and co-workers (118) found that both domains were necessary. We examined the ability of R2-H42Q and R2-E109K cells to form colonies in soft agarose (Table 2). Parental Rat 2 cells and M1R2/7 cells, a JCV-transformed Rat 2 line obtained in a dense focus assay (8), were included as negative and positive controls, respectively. Cells selected on the basis of their transformed phenotype (M1R2/7) readily formed large colonies (14%), whereas cells expressing an intact early region (R2-JCV_E), formed colonies with lower efficiency (5%). The E109K cell line was unable to form colonies, indicating that the RB-binding domain is required for AIG. However, cells expressing H42Q mutant proteins did grow in soft agarose (2%), albeit less well relative to cells producing JCV WT early proteins.
Table 2: AIG properties of Rat 2 cells expressing JCV WT and mutant early proteins.

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>% Cell forming Colonies</th>
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<tbody>
<tr>
<td>M1R2/7</td>
<td>14</td>
</tr>
<tr>
<td>R2-JCV/E</td>
<td>5</td>
</tr>
<tr>
<td>R2-H42Q</td>
<td>2</td>
</tr>
<tr>
<td>R2-E109K</td>
<td>0</td>
</tr>
<tr>
<td>Rat 2</td>
<td>0</td>
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*a* AIG of G418 selected Rat 2 cells expressing JCV with WT (R2-JCV/E), LXCXE domain mutant (R2-E109K) or J domain mutant (R2-H42Q) early proteins were compared to that of parental Rat 2 cells and a JCV transformed line (M1R2/7).

*b* Colonies larger than 0.05 mm were counted in 20 randomly selected fields from two 60 mm dishes and percentage of cells developing into colonies was calculated.
2.5 DISCUSSION

The large (TAg) and small (tAg) T antigens of SV40 have been studied extensively and are known to possess oncogenic activity. The role of SV40’s third early protein, 17KT, in transformation is largely unknown. The key transforming functions of TAg relate to its interactions with, and inactivation of, the cellular tumor suppressor proteins p53 and pRB. SV40 TAg inactivates the “gatekeeper” function of the RB family members and promotes cell proliferation via the release of members of the E2F family of transcription factors (96, 112, 135). In previous work we demonstrated that JCV T’ proteins interact with pRb, p107 and p130 in vitro and in vivo (9); Bollag et al., unpublished data), and contribute to viral DNA replication (81, 124). Recent reports have identified the presence of JCV and SV40 sequences in human tumors, and it is critical to expand studies involved in understanding the oncogenic potential of these polyomaviruses to include functional analyses of the TAg isoforms. The current study focused upon the outcome of the interactions between the JCV tumor proteins and members of the RB family of tumor suppressor proteins.

Mutations in the LXCXE and J domains of JCV early proteins.

The analyses of cell lines stably expressing E109K or H42Q mutant proteins indicate that the LXCXE and J domains contribute to the release of E2F-1 and E2F-4 from RB-E2F complexes. 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 LXCXE motif fail to degrade 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 Hsc70’s intrinsic ATPase activity, thereby disrupting the complex and releasing E2F (96, 112). JCV H42Q mutant proteins exhibit the ability to interact with both the hyperphosphorylated and hypophosphorylated forms of p107 and p130, but fail to reduce the levels of hyperphosphorylated forms of p107 and p130 in the R2-H42Q line. As reported earlier for SV40 TAg (10, 109, 110), our findings suggest that the J domain of JCV tumor proteins contributes to the disappearance of hyperphosphorylated forms of p107 and p130. Further, the WT and H42Q mutant proteins appeared to differ in their ability to interact with hyperphosphorylated p107 and p130.

Studies using SV40 TAg show the half-life of p130 is reduced from 5 hrs to 1 hr in the presence of WT TAg. However, in the presence of TAg J domain mutant H42Q, the half life of p130 increased to 10 hrs. (22). Similarly, our data shows that WT JCV early proteins alter the phosphorylation status of p130 in cells and send the hyper-phosphorylated form for rapid degradation via the 26S proteosome. Further, similar to SV40 J domain mutant, JCV mutant H42Q also blocked the degradation of p130 (Figure 2.1).

**Differential release of E2F proteins from RB-E2F complexes.**

Recently, a two step model for E2F-dependent transcription was proposed, wherein repressive E2F complexes are dislodged from promoters preceding activation of the transcriptional element by activator E2Fs (75). Because we have observed differences in the binding of JCV TAg and T' proteins to RB proteins, we tested the prediction that
the viral proteins differ in their ability to regulate both activating (E2F-1) and repressive (E2F-4) classes of E2F members. We found the steady state levels of E2F-1 and E2F-4 were similar in cells expressing different JCV early proteins, but the cellular localization of E2F-4 varied in these same cell lines. Sullivan et al. (111) reported that SV40 infection of permissive cells disrupts p130-E2F complexes, leading to the relocalization of E2F-4 from the nucleus to the cytoplasm. These investigators also noted that steady state levels of E2F-1 were unchanged in the infected cells, and that both RB-bound and free forms of E2F-1 were present in the nucleus.

Harris et al., (42) used an electromobility-gel shift assay to quantitate free and complexed forms of E2F in cells expressing BKV TAg or SV40 TAg and found only modest 1.6 or 1.8 fold increases in the free form. Our initial attempts to measure the two forms of E2F-1 and E2F-4 in our cell lines by this assay were inconclusive. Because numerous groups have employed reporter assay systems to quantitate free transcriptionally-active E2F (42, 43, 96), (2), we developed a luciferase assay to demonstrate that TAg and T' proteins cause the release of different amounts of transcriptionally-active E2F-1 and E2F-4 (for statistical significance refer to Table 3). In an earlier study (9) it was determined that T'165, when compared to T'135 and T'136, interacts more efficiently with human pRb and less efficiently with human p107 and p130. In the current study, interaction of TAg and T' proteins with pRb could not be established in rat cells due to low levels of pRb expression. However, we did show that T'165 effects the release of E2F-1, a predominant binding partner of pRb (reviewed by (25), more efficiently than the other JCV early proteins. In contrast, T'165 has little or no effect on the release of E2F-4, which primarily associates with p107 and p130 (116) and
references therein). Relative to $T_{165}'$, $T_{135}'$ demonstrated the opposite effect on E2F release; its influence on the release of E2F-4 was more pronounced than its effect on E2F-1. $T_{136}'$, which differs from $T_{135}'$ only in its C-terminal four amino acids, had a modest effect on both E2F-1 and E2F-4 release. An additive effect of the individual JCV early proteins on E2F-1 release was not observed in cells expressing the intact JCV early region (R2-JCV$_E$). It is possible that the individual T' proteins act independently of each other in the context of E2F release.

Minimal E2F activity was measured in cells expressing TAg in the absence of other JCV early proteins (R2-TAg cells). Although we could not rule out the possibility that this finding was due to the low levels of TAg expressed in this line, results from transient transfection experiments support our conclusion that the JCV TAg does not effectively release E2F transcription factors complexed to RB family members. Other groups have reported that SV40 TAg disrupts RB-E2F complexes (96, 111, 112), although each study used constructs that presumably expressed 17kT as well as TAg. Recently, Boyapati et al. (10) reported that SV40 17kT is capable of promoting p130 turnover and stimulating quiescent cells to enter the cell cycle. The significance of this observation in relation to TAg function was not fully explored due to the investigators’ inability to generate a virus that expressed TAg in the absence of 17kT. In a related study of BKV, Harris et al. (42) failed to detect an interaction between TAg and RB family members, yet observed E2F activation in cells expressing BKV early proteins. Although it was suggested that these effects resulted from transient interactions between BKV TAg and RB proteins, it is possible that a BKV TAg isoform (mini-T protein; Bam, Prins and
Frisque, unpublished data) contributes to the reduction in the levels of cellular RB proteins and activation of E2F.

Table 3: Dunnett simultaneous tests using general linear model to test for statistical significance in the E2F values.

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>T-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMV-JCV_E</td>
<td>6.3199</td>
<td>0</td>
</tr>
<tr>
<td>CMV-SV40_E</td>
<td>2.2786</td>
<td>0.1461</td>
</tr>
<tr>
<td>CMV-T'_{135}</td>
<td>1.5206</td>
<td>0.5336</td>
</tr>
<tr>
<td>CMV-T'_{136}</td>
<td>3.5287</td>
<td>0.0066</td>
</tr>
<tr>
<td>CMV-T'_{165}</td>
<td>12.4859</td>
<td>0</td>
</tr>
<tr>
<td>CMV-TAg</td>
<td>0.0435</td>
<td>1</td>
</tr>
<tr>
<td>CMV-H42Q</td>
<td>1.5876</td>
<td>0.487</td>
</tr>
<tr>
<td>CMV-E109K</td>
<td>0.1266</td>
<td>1</td>
</tr>
</tbody>
</table>

To investigate the downstream effects of E2F release from RB-E2F complexes, we followed the progression of cells expressing JCV proteins through the cell cycle. Cells were growth arrested by serum starvation for 72 hours, then stimulated with medium containing 10% FCS. Cells expressing the five early proteins (R2-JCV_E) entered S phase sooner than cells expressing individual T' proteins, suggesting that the coordination of early protein activities is necessary to efficiently promote this progression. We propose that JCV T'_{135} disrupts repressor complexes targeted to promoters of S phase genes, and that T'_{165} facilitates a shift of activator E2Fs to these promoters to induce cell cycle entry and progression. With T' proteins regulating the RB-E2F pathway, TAg and tAg would
be free to target other critical signaling molecules, including p53 and PP2A, thus optimizing conditions for viral replication and virus-host interactions (Figure 2.17).

**Model: JCV T' Proteins & Cell Cycle Progression**

**A**

\[\text{Promoter} \]

- p130
- E2F-4

**B**

\[\text{Cytosol} \]

- T'_135
- E2F-4
- p130

\[\text{Nucleus} \]

- T'_135
- E2F-4
- Degradation

**C**

\[\text{Promoter} \]

- T'_165
- E2F-1
- pRb
- p107
- E2F-1

**D**

\[\text{Replication} \]

- TAg

\[\text{Transcription} \]

- RF/TF

\[\text{Prevent Apoptosis} \]

- p53
- PP2A

\[\text{Proliferation} \]

Figure 2.17: Proposed model for the role of JCV early proteins in S phase progression.

(A) A p130/E2F-4 complex is bound to the promoter of S phase genes thereby repressing transcription. (B) T'_{135} binds to and disrupts the p130/E2F-4 complex, thereby relocating E2F-4 to the cytoplasm and sending ubiquitinated (Ub) p130 for degradation via the 26S proteosome. (C) T'_{165} interacts with pRb/E2F-1 and p107/E2F-1 complexes to release E2F-1, which now occupies the E2F-4 free promoter to activate transcription of S phase genes. (D) Apoptosis activated by unscheduled cellular proliferation induced by T' proteins may be blocked by a TAg-p53 interaction. Small tAg may contribute to cellular proliferation and/or prevent apoptosis following its interaction with the serine/threonine phosphatase PP2A (25, 37).

Finally, we have examined the requirements for functional LxCxE and J domains in the JCV early proteins in AIG and DNA replications assays. In some of our experiments the H42Q mutation appeared to be “leaky.” Although the mutant proteins were defective in reducing the levels of hyperphosphorylated RB proteins in R2-H42Q
cells, they exhibited partial release of E2F proteins from RB-E2F complexes, observations similar to those made with the corresponding SV40 H42Q mutant (29). In our attempt to make J domain fully non-functional we introduced another mutation into the HPD motif to create the double mutant (H42Q/D44N). In a transient co-transfection assay the double mutant exhibited reduced luciferase activity, but this activity was still greater than that observed with the control vector (Figure 2.162). We also noted that cells expressing the H42Q mutant formed colonies in the AIG assay, albeit with reduced efficiency relative to the JCV line; the R2-E109K cells failed to grow under these conditions. It is unclear whether our AIG assay results reflect leakiness of the H42Q mutation, since there are conflicting reports regarding the requirement for an intact J domain in this assay using SV40-transformed cells. Tevethia and co-workers (118) found that both WT J and LXCXE domains were necessary for AIG, whereas Stubdal et al. (109) concluded that only the latter domain was required.

In agreement with SV40 replication studies (14), we found that the J domain of JCV early proteins is required for efficient viral DNA replication. We are unaware of any studies that have examined the DNA replication potential of SV40 LXCXE mutants, however, we determined that the E109K mutation reduced, but did not abolish JCV replication activity.

Identification of three TAg isoforms reveals an unexpected complexity in the JCV genome and suggests that assumptions about TAg’s contributions to viral lytic and transforming behavior should be reassessed. A number of recent studies examining the association between polyomaviruses and apoptosis, cell cycle progression, transformation and immortalization, have limited their analyses to TAg and tAg functions; the
contributions of TAg isoforms have not been evaluated. To gain a more complete understanding of the oncogenic and pathogenic potential of this family of viruses, it is important to understand the functions of these “truncated” TAg isoforms. Our observations suggest that JCV utilizes its three T' proteins to deregulate the RB pathway, while engaging its TAg in other host cell interactions to promote viral replication or transformation.
Chapter 3

THE STABILITY AND FUNCTION OF JCV TAg AND T’ PROTEINS ARE ALTERED BY MUTATION OF THREONINE 125 RESIDUE.
3.1 ABSTRACT

JC virus (JCV), a human polyomavirus, exhibits oncogenic activity in rodents and primates. The large tumor antigen (TAg) of the polyomaviruses plays key roles in viral replication and oncogenic transformation. Analyses of JCV TAg phosphorylation mutants indicate that the amino-terminal phosphorylation site at threonine 125 (T125) is critical to TAg replication function. This site is also conserved in the TAg splice variants T'$_{135}$, T'$_{136}$ and T'$_{165}$. By constructing stable cell lines expressing the JCV mutants T125A and T125D, we show that mutation of the threonine residue to an alanine generates an unstable TAg; stability of the three T' proteins is unaffected. JCV T125A proteins bind RB family members, p107 and p130, with reduced efficiency, and fail to induce the release of transcriptionally-active E2F from RB-E2F complexes. On the other hand, cell lines expressing JCV T125D proteins produce stable TAg and T' proteins which bind p107 and p130 more efficiently than do the wild type proteins. In addition, T125D mutant proteins efficiently induce the release of E2F from RB-E2F complexes. T125D cell lines, unlike the T125A lines, continue to grow under conditions of low serum and anchorage-independence. Finally, both T125A and T125D mutant viruses are replication-defective. Phosphorylation of the T125 site is likely mediated by a cyclin:cyclin-dependent kinase, suggesting that JCV TAg and T' protein functions that mediate viral replication and oncogenic transformation events are regulated in a cell cycle-dependent manner.
3.2 INTRODUCTION

Human polyomavirus JC virus (JCV) may persist in the kidneys and brain of healthy individuals at subclinical levels, but may cause the fatal demyelinating disease progressive multifocal leukoencephalopathy (PML) in immunocompromised patients. Members of the Polyomaviridae family replicate in actively dividing cells because they rely upon the host cell’s machinery to copy their DNA and produce infectious virions (61). The best studied of these viruses, simian virus 40 (SV40), shares 69% sequence similarity with JCV. Both viruses produce a multifunctional T protein (TAg) that mediates viral DNA replication and promotes the transition of cells from G₀ into S phase (24). Depending on the cell type, this latter event may either support a lytic infection of permissive cells or contribute to transformation of non-permissive cells.

Hypophosphorylated forms of RB pocket proteins bind members of the E2F family of transcription factors, thereby providing a gatekeeper function that prevents unscheduled cell cycle progression. The RB-E2F complexes regulate genes that control entry into, and progression through S phase, and they coordinate these cell cycle programs via regulation of checkpoint controls, DNA damage responses, apoptosis and differentiation (reviewed by (19) references therein). Upon receipt of mitogenic signals, cells express cyclin proteins that interact with specific cyclin-dependent kinases (Cdk) to promote cell cycle transition. Cyclin:Cdk complexes phosphorylate pRb, p107 and p130, thereby causing the release of their E2F partners which activate expression of S-phase genes (reviewed by (98), (78). DNA tumor virus proteins, including TAg, override normal cell cycle regulation by binding to RB proteins and displacing the E2F proteins,
resulting in far wider consequences than simply increasing cellular proliferation (reviewed by (19) references therein).

Polyomavirus TAgS have multiple functional domains that support their roles in promoter activation and viral DNA replication. SV40 TAg has two phosphorylation domains containing eight or more sites clustered between serine 106 (S106) and threonine 124 (T124) at its amino terminus and S639 and T701 at its carboxy terminus (92). Mutations to the carboxy-terminal sites affect phosphorylation at the amino-terminal sites (91). Post-translational modifications of SV40 TAg modulate its DNA binding (99), replication (73) and p53 binding (15) activities. Phosphopeptide mapping of JCV TAg also revealed two phosphorylation domains (114), and a mutation to T125 abolishes viral DNA replication (115). To initiate viral DNA replication, monomeric TAg oligomerizes to form hexamer and double hexamer complexes in the presence of ATP, and these TAg structures bind the viral origin of replication (99). The formation of the first SV40 TAg hexamer is independent of the phosphorylation status of the critical T124 residue. However, mutation of this residue to an alanine (T124A) disrupts formation of the second SV40 TAg hexamer without preventing specific DNA binding activity. Further, mutation of T124 to an aspartic acid (T124D) does not affect double hexamer formation, but DNA binding is inefficient. These results suggest that T124 phosphorylation leads to changes in TAg-DNA interactions to facilitate structural changes required for hexamer formation (50) and references therein). Phosphorylation-dependent regulation of TAg is thought to be cell cycle regulated, with TAg hexamerization occurring as the host cell enters S phase. McVey et al., (69) reported that a Cdk purified from human cells phosphorylates the T124 residue in vitro. This
modification inhibits nuclear import of SV40 TAg \textit{in vivo}; a similar effect is observed with the T124D mutant (48).

The present chapter details our investigation of two mutants altered at amino acid residue 125 of the JCV TAg and the 3 TAg isoforms, T'\textsubscript{135}, T'\textsubscript{136} and T'\textsubscript{165}. The mutation of T125 to an alanine (T125A) alters the stability of JCV TAg and the function(s) of the early viral proteins. The three T' proteins with the T125A mutation exhibit reduced binding affinity for the RB family members p107 and p130. Mutation of the same residue to an aspartic acid (T125D) yields JCV early proteins that exhibit stronger interactions with the two pocket proteins. As predicted from these results, the release of E2F-1 and E2F-4 from RB-E2F complexes is influenced by the T125 mutations. Furthermore, Rat 2 cells expressing the T125D mutant proteins exhibit growth under conditions of anchorage–independence and low serum. Localization studies reveal that the JCV T125D TAg, unlike its SV40 counterpart, is targeted to the nucleus. Both the T125A and T125D mutants are replication-defective in human cells. We propose that phosphorylation of the T125 site is essential to JCV TAg stability and to the coordination of JCV early protein functions required for productive viral replication and oncogenic transformation.
3.3 MATERIALS AND METHODS

Construction and sources of expression vectors.

BS/JCV(T125A) was created by ligating the JCV(T125A) genome from the recombinant pJCV-T125A clone (115) into the Bluescript II SK (-) vector using an EcoRI restriction site. CMV-JCV\text{E} (T125A) was constructed by swapping a PflMI-BglII fragment containing the T125A mutation from BS/JCV(T125A) into CMV-JCV\text{E}, a construct that expresses the entire JCV early region via the CMV early promoter (Bollag et al., in preparation). BS/JCV(T125D) was created using a PCR-based site-directed mutagenesis approach (Stratagene). CMV-JCV\text{E} (T125D) was created by swapping a PflMI-BglII fragment containing the T125D mutation from BS/JCV(T125D) into CMV-JCV\text{E}. Mutations were introduced using primers T125Afwd 5′-GGATCCCAACACTCTGGCCCACCTAAAAAG-3′, T125Arev 5′-CTTTTAGGTGCGAGAGTGTTGGG-3′, T125Dfwd 5′-CCCAACACTCTGACGGGGGATCCCTATAAAAG-3′ and T125Drev 5′-CTTTTAGGTGGTGAGGTGTTGGG-3′ (mutations are in bold, underline). Each DNA construct was confirmed by sequence analysis.

Luciferase reporter plasmids containing either 4 copies of the E2F-1 promoter element (pE2F1-luc) or 4 copies of the E2F-4 promoter element (pE2F4-luc), and an isogenic construct lacking E2F promoter elements (ΔE2F-luc) were kind gifts from Dr. J. R. Nevins (Duke University). A β-gal expression vector under the control of a CMV promoter (pCMV-βgal) was obtained from Dr. G. H. Perdew (Penn State University).
**Cell Culture.**

Rat 2 cells expressing various JCV WT and mutant proteins, R2-JCV\textsubscript{E}, R2-T125A, R2-T125D, R2-T\textsuperscript{′}135, R2-SV40\textsubscript{E} and R2CT (G418-selected Rat 2 cells) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% bovine calf serum (BCS), 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin at 37°C under 10% CO\textsubscript{2}. PHFG cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate and 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin at 37°C under 10% CO\textsubscript{2}.

**G418 selection.** The cell lines R2-T125A and R2-T125D were created by transfecting Rat 2 cells with 1 µg CMV-JCV\textsubscript{E} (T125A) or CMV-JCV\textsubscript{E} (T125D) DNA using Lipofectamine 2000 reagent (Invitrogen). Transfected cells were propagated in DMEM containing 400 µg/ml geneticin sulfate and supplemented with 10% BCS. Surviving cells exhibiting G418 resistance were isolated at 10 days post-transfection (p.t.) and subcloned by limiting cell dilution.

**Transient Transfections and Lucifase Reporter Gene Assay.**

To test transcriptionally active free E2F in cells, R2CT cells (2.5x10\textsuperscript{5}) were grown in 6-well culture dishes containing DMEM supplemented with 10% BCS, 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin at 37°C in 10% CO\textsubscript{2}. Cells were co-transfected 10-12 hrs post plating, with 750 ng pE2F1-luc or pE2F4-luc, 500 ng pCMV-βgal, and different concentrations of JCV TAg and T\textsuperscript{′} DNA constructs using lipofectamine 2000 reagent (Invitrogen). Cells were transfected for 3 hrs in serum and antibiotic free media as per the manufacturer’s recommendations. The luciferase activity
was assayed 48 hrs post transfection using a Turner TD-20e luminometer with a dual luciferase assay system (Promega), and luciferase readings were normalized to β-gal activity.

**Immunoprecipitation and Western Blot Analysis.**

Protein-protein interactions were demonstrated by a co-immunoprecipitation assay consisting of IP and WB steps. The cells were lysed in EBC buffer (50 mM Tris, pH 8.0, 120 mM NaCl, 0.5% NP-40) containing protease and phosphatase inhibitors (10 µg/ml leupeptin, 10 µg/ml aprotinin, 10 µg/ml E-64, 1 mM pefabloc, 5 mM sodium fluoride, 1 mM sodium vandadate, 500 mM EDTA). The IP step involved adding specific anti-T or anti-RB antibodies to cell lysates prepared in EBC buffer and proceeding as described earlier (123). The WB step involved electrophoresing the complexes on SDS polyacrylamide gels, transferring the separated proteins to membranes, adding anti-RB or anti-T antibodies to the membrane and adding a second antibody to permit visualization of protein bands (123). The unstable TAg in R2-T125A cells was stabilized by treating the cells with the proteosome inhibitor, MG-132 (10 µM), and incubating for 4 hrs at 37°C before preparing the cell lysate in EBC buffer containing protease inhibitors.

IP/WB analysis was conducted using the following primary antibodies: anti-T (PAb 962 (118), PAb 2001 and PAb 2023 (9), anti-p107 (c-18; Santa Cruz) and anti-p130 (c-20; Santa Cruz). Anti-mouse IgG alkaline phosphatase conjugate (A-3562; Sigma) was used as secondary antibody.
**Pulse Chase Analysis.**

R2-JCV<sub>E</sub> and R2-T125D (5x10<sup>5</sup>) cells were seeded into 35 mm dishes containing DMEM and 10% FBS. Twelve hrs later, medium was replaced with DMEM containing 200 µCi of <sup>35</sup>S-methionine, 5% FBS, 1 mM sodium pyruvate and 2 mM L-glutamine for 5 hrs at 37°C under 10% CO<sub>2</sub>. After 5 hrs, the cells were washed twice in phosphate buffer saline (PBS) and replaced with DMEM supplemented with 10% FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin and incubated at 37°C under 10% CO<sub>2</sub>. Cell lysates were prepared at 0, 6, 12, 24, 36 and 48 hr time intervals using EBC buffer containing protease and phosphatase inhibitors. TAg was immunoprecipitated with antibody PAb 962 and electrophoresed on a 15% SDS-polyacrylamide gel. The protein bands were detected by autoradiography and band intensities were quantitated using ImageQuant 5.2 software (Molecular Dynamics).

**In Vitro Transcription/Translation.**

The TAg cDNA containing the T125A mutation was used to create the protein product *in vitro* using TnT coupled reticulocyte lysate system (Promega). The reaction was set up according to the procedure described in the manufacturer’s manual. After the reaction was finished 10µl of the final product was loaded on 8% SDS gel for analysis. The TAg labeled with <sup>35</sup>S-methionine was observed by autoradiography and the migration of T125A mutant protein was compared with that of WT to look for size difference.
**RT-PCR Analysis.**

Total cellular RNA was isolated from R2CT, R2-JCV\_E and R2-T125A cells using the RNeasy mini kit (Promega). After DNAase treatment the isolated RNA was subjected to reverse transcription-polymerase chain reaction (RT-PCR) analysis using the Access RT-PCR System (Promega) with primers (nucleotides 5019-4993) T\#5 5\' -GC TAAAATGGACAAAGTGCTGAATAGG- 3\' and (nucleotides 4249- 4274) T\#6 5\'-CAGGAAAGTCTTTAGGG TCTTCTACC- 3\'.

**DpnI Replication Assay.**

PHFG cells were seeded onto 60 mm dishes and transfected the following day with JCV DNA (400 ng) using Lipofectamine 2000 reagent according to the manufacturer’s protocol. Low molecular weight DNA was isolated from the cells at 0, 7, 10 and 14 days p.t. by the Hirt extraction procedure (45). A portion of the DNA was cleaved with the restriction enzymes DpnI and EcoRI, and digestion products were separated by electrophoresis on a 0.8% agarose gel. DNA fragments were transferred to a nylon membrane using a Rapid Downward Transfer System and an alkaline transfer protocol (Schleicher and Schuell, Amersham). The immobilized samples were hybridized with linear full-length JCV DNA labeled with [\(\alpha^{32}\)]dCTP (oligo labeling kit, Pharmacia). Relative replication activities of different viral DNAs were determined by quantitating band intensities using ImageQuant 5.2 software (Molecular Dynamics).
**Anchorage-Independent Growth (AIG) Assay.**

Parental and G418-resistant Rat 2 cells were suspended in DMEM containing 10% FBS plus 0.25% agarose and seeded onto 60 mm dishes coated with a layer of 0.5% agarose medium. Three dishes were seeded with cells from each line (1×10^5 cells/dish). Fresh medium was added to the plates every 7 days, and after 3 weeks, colonies with diameters exceeding 0.5 mm were counted in 20 to 30 randomly-selected fields. The percentage of cells developing into colonies was then calculated (8).

**Cell Cycle Analysis.**

Cells (2.2×10^5) were seeded onto 60 mm dishes in DMEM containing 0.01% BCS. After 72 hrs the cells were stimulated with medium containing 10% BCS. Cells were removed from the dishes with trypsin at 0, 9, 12 and 15 hrs post-stimulation and pelleted. After washing in PBS, cells were pelleted and resuspended in 300 µl PBS. Cells were fixed in 300 µl 70% ethanol, and after 2 hrs the cells were pelleted, washed in PBS and pelleted once more. Cells were suspended in 500 µl PI buffer (PBS containing 0.1% Triton X-100, 1 µg/ml RNAse, 20 µg/ml propidium iodide). Samples were incubated at 37°C for 15 minutes and then subjected to flow cytometric analysis (XL-MCL Coulter machine at the Center for Quantitative Cell Analysis, Penn State University).

**Immunofluorescence.**

R2CT, R2-JCV\textsubscript{E}, R2-T125A and R2-T125D cells were plated at low density on glass cover slips. The cells were fixed with a 1:1 mixture of methanol and acetone for 10 minutes, and then incubated for 45 minutes with 12 µl of anti-T antibodies (PAb 962,
PAb 2001 and PAb 2023) diluted 1:20. After washing the cover slips in PBS three times, the cells were incubated with fluorescein-conjugated mouse immunoglobulin G serum for 45 minutes. The cells were observed and photographed using an Olympus BX-60 epi-fluorescent upright microscope with a Hamamatsu Orca-100 camera (Center for Quantitative Cell Analysis, Penn State University).

3.4 RESULTS

Derivation of cell lines expressing T125A and T125D mutant proteins.

Mutation of T124 in SV40 TAg or T125 in JCV TAg to an alanine results in a replication-defective virus (93, 114). These mutations affect not only TAg, but also the SV40 and JCV TAg isoforms, 17KT, T'\(_{135}\), T'\(_{136}\) and T'\(_{165}\); small t antigen (tAg) does not contain the mutation. To investigate the replication defect in the JCV T125A mutant, CMV-JCV\(_E\) (T125A) DNA was transfected into Rat 2 cells to establish a G418-resistant cell line that expressed the mutated JCV early region. Independent clones were screened to identify cells expressing the 5 JCV early proteins. Nine lines containing JCV proteins were obtained, but surprisingly, none of them expressed detectable levels of TAg (Figure 3.51). The other four early proteins were detected, however, the TAg isoforms, T'\(_{135}\), T'\(_{136}\), T'\(_{165}\), which normally migrate as doublets in dividing cells due to phosphorylation (Bollag et al., in preparation), were each represented by a single hypophosphorylated band that migrated at the same position as the phosphatase-treated protein bands derived from the wild type (WT) JCV\(_E\) control cells.
An aspartic acid residue was introduced at the T125 site to create the expression plasmid, CMV-JCV_E (T125D). Based upon earlier studies (48), we predicted that this substitution would mimic the negative charge of a phosphorylated threonine residue in the WT TAg. CMV-JCV_E (T125D) DNA was transfected into Rat 2 cells to establish a G418-resistant cell line. Six lines expressing JCV proteins were screened to determine the protein expression level. Introduction of a negative charge at amino acid 125 stabilized TAg expression, and the protein was readily detected in our IP/WB analysis (Figure 3.1). The three mutant T' proteins migrated as single bands and thus shared this property with the T125A mutant proteins.
Figure 3.1: Rat 2 cells expressing JCV\textsubscript{E} T125A and T125D mutant proteins.

Whole cell extracts were prepared from subconfluent R2-JCV\textsubscript{E}, R2-T125A and R2-T125D cells, and proteins were immunoprecipitated with PAb 962 antibody. Co-immunoprecipitated proteins were separated on a 15% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane and probed with PAb 962, PAb 2003 and PAb 2023 antibodies. Anti-rabbit IgG alkaline phosphatase conjugated antibody was used as the secondary antibody, and protein bands were visualized using NBT/BCIP substrate buffer solution. The JCV\textsubscript{E}* sample was treated with lambda protein phosphatase after the IP reaction. This figure is a composite and the center of the blot has been digitally removed to show only the relative viral protein bands.
**Status of TAg in R2-T125A cells: mRNA and protein analyses.**

The lack of TAg expression in R2-T125A cells led us to speculate that either TAg mRNA was rapidly degraded or the translated protein was highly unstable in R2-T125A cells. To address these possibilities, steady state levels of TAg mRNA were examined in R2-JCV<sub>E</sub> and R2-T125A cells by RT-PCR (Fig. 3.2A). TAg mRNA was readily detected in both lines, and the transcripts were the same size, suggesting no major alterations to RNA processing had occurred. I then treated R2-T125A cells with the 26S proteosome inhibitor MG-132 and looked for TAg expression by IP/WB analysis (Figure 3.2). After MG-132 treatment, the T125A TAg was detected, although it migrated as a 42 kD, rather than as a 92 kD, protein on the gel. The anti-T monoclonal antibody used in this experiment recognizes an epitope within the amino terminus of TAg. Therefore, the experiment was repeated using a second monoclonal antibody, PAb 901, which recognizes carboxy-terminal sequences. No TAg fragments were detected using this approach (data not shown).
Figure 3.2: Analyses of viral mRNAs and proteins in R2-T125A cells.

(A) RT-PCR analysis of viral mRNA in R2-T125A cells. Total cellular RNA was collected from R2CT, R2-JCV<sub>E</sub> and R2-T125A cells, treated with DNase and subjected to RT-PCR analysis using primers T′#5 and T′#6. (B) IP/WB analysis of TAg in R2-T125A cells. Whole cell extracts were prepared from subconfluent R2-JCV<sub>E</sub> and R2-T125A cells, the latter cells were treated with the proteosome inhibitor MG-132 (10 µM) for 4 hrs at 37°C before preparing the cell lysate. Proteins were immunoprecipitated with PAb 962 antibody. Co-immunoprecipitated proteins were separated on a 15% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane and probed with PAb 962, PAb 2003 and PAb 2023 antibodies. Anti-rabbit IgG alkaline phosphatase conjugate antibody was used as the secondary antibody, and protein bands were visualized using NBT/BCIP substrate buffer solution. T* indicates the position of a 42 kD amino-terminal fragment of TAg.
In-vitro transcription/translation of TAg (T125A) mutant.

Detection of unstable TAg (T125A) mutant after treatment of cells with MG-132 suggested that TAg was being degraded via the 26S proteosome. The ability to rescue the N-terminal 42 kDa fragment of TAg but not the C-terminal fragment, suggested a regulatory step that differentiated TAg carboxy from the amino terminus. To gain a better understanding of this regulatory step, a TnT coupled reticulocyte lysate system was used to transcribe and translate TAg T125A mutant DNA in vitro to produce a full length protein. This full length protein was then incubated in a Rat 2 cell lysate to test for protein instability. The stable TAg was collected using an IP assay with PAb 962. Protein stability was examined by electrophoresing the in vitro transcribed and translated WT TAg and TAg T125A mutant products in an 8% SDS gel (Figure 3.3). Incubation of TAg T125A in cell lysate did not detectably alter the stability of the protein.
Figure 3.3: In vitro transcription/translation of TAg (T125A) cDNA.

Stability of TAg T125A was tested using TnT coupled reticulocyte lysate *in vitro* transcription and translation system. The *in vitro* TAg product was incubated in Rat 2 whole cell extracts either on ice or at 37°C. After 1.5 hrs TAg stability was examined by immunoprecipitating TAg using PAb 962 and resolving the protein on an 8% SDS gel. Lanes 1 and 4 shows no size difference between *in vitro* produced WT TAg and the TAg T125A mutant. Lanes 2 and 5 shows that WT and T125A mutant TAg’s are both stable after incubation in Rat 2 cell extract on ice for 1.5 hrs. Lanes 3 and 6 indicate that WT and mutant TAg’s are also stable when incubated in Rat 2 lysate at 37°C. ³⁵S-methionine labeled TAg protein bands were observed by autoradiography.
Do TAg and T' proteins physically interact?

R2-T125A cells contain a labile TAg and three T' proteins that lack the T125 phosphorylation site. It is possible that JCV TAg is stabilized by direct interaction with a WT T' protein; such an interaction(s) would also be expected to influence other TAg and T' protein functions. To test this possibility, JCV TAg purified from insect cells infected with a recombinant baculovirus-TAg vector was incubated with extracts of rat cells stably expressing T'135 or T'136. (Bollag et al., manuscript in preparation). A co-IP experiment was conducted using PAb 901 for the IP step and PAb 962 for the WB step. The first monoclonal antibody recognizes TAg but not T'135 nor T'136, and the second antibody recognizes all three early proteins. Co-immunoprecipitation of T'135 or T'136 with TAg was not observed (Figure 3.4). The IP antibody PAb 901 does recognize the third T' protein, T'165, therefore a different approach will be required to see if this protein forms a complex with TAg.
Figure 3.4: TAg does not interact detectably with T' proteins.

The first 3 lanes show the amounts of individual proteins (TAg, T'_{135}, T'_{136}) used to examine potential TAg-T' protein interactions. Lanes 4 and 5 show purified TAg (1µg) incubated with sub-confluent R2-T'_{135} and R2-T'_{136} whole cell extracts, and proteins immunoprecipitated with PAb 901 antibody. Co-immunoprecipitated proteins were separated on a 15% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane and probed with PAb 962 antibody. Anti-rabbit IgG alkaline phosphatase conjugate antibody was used as the secondary antibody, and protein bands were visualized using NBT/BCIP substrate buffer solution. No interactions were observed in the TAg + T'_{135} and TAg + T'_{136} lanes.
The amino-terminal 42 kD TAg fragment stabilized by MG-132 localizes to the cell nucleus.

The nuclear localization signal (NLS) in SV40 TAg (amino acids 126-131) is positioned near the T124 residue, and mutation of the latter residue inhibits NLS function (48). A similar arrangement occurs in the JCV TAg, but because the JCV T125A TAg mutant is unstable, we could not directly determine whether the mutation affected TAg subcellular localization. Therefore, we treated R2-T125A cells with MG-132 to yield a stable amino-terminal TAg fragment. Cells expressing WT and mutant JCV TAg were examined by indirect immunofluorescence using antibody PAb 962 (Figure 3.5). No nuclear staining was observed in parental Rat 2 cells or in the untreated R2-T125A line. Nuclear fluorescence was detected in R2-JCV_E and R2-T125D cells. Interestingly, punctate nuclear TAg staining was seen in R2-T125A cells treated with MG-132, indicating a functional NLS in the 42 kD TAg fragment.
Figure 3.5: Nuclear localization of T125A and T125D mutant TAgS.

R2CT, R2-JCV\textsubscript{E}, R2-T125D and R2-T125A (+/- MG-132 treatment) cells were plated at low density on glass cover slips. The cells were fixed with a 1:1 mixture of methanol and acetone for 10 minutes, and then incubated for 45 minutes with 12 µl of anti-T antibodies (PAb 962, PAb 2001 and PAb 2023) diluted 1:20. After washing the cover slips in PBS three times, the cells were incubated with fluorescein-conjugated mouse immunoglobulin G serum for 45 minutes and observed under microscope.
**Stabilities of WT and T125D TAg are similar.**

The T125A mutation resulted in a labile JCV TAg. To test if the negative charge at position 125 increased the stability of the T125D TAg, R2-JCV\textsubscript{E} and R2-T125D cells were labeled in medium containing \textsuperscript{35}S-methionine-containing medium for 5 hrs and then refed with DMEM without \textsuperscript{35}S-methionine. Lysates were collected at regular intervals and analyzed by IP (Figure 3.6). Although TAg was labeled more efficiently in R2-T125D cells compared to that in R2-JCV\textsubscript{E} cells, quantitation of band intensities over the time course of the experiment indicated that the WT and mutant TAg had similar stabilities.
**Figure 3.6:** The stabilities of WT and T125D mutant TAgS are similar.

R2-JC\textsubscript{E} and R2-T125D (5\texttimes{}10\textsuperscript{5}) cells seeded into 35 mm dishes were pulsed with 50 µci of \textsuperscript{35}S-methionine for 5 hrs. The cells were washed twice in PBS and refed with DMEM containing 10% FBS. At 0, 6, 12, 24, 36 and 48 hrs post-pulse, cells were lysed in EBC buffer containing protease and phosphatase inhibitors. TAg was immunoprecipitated using antibody PAb 962 and electrophoresed on a 15% SDS-polyacrylamide gel. Protein bands were detected by autoradiography and band intensities were quantitated using ImageQuant 5.2 software (Molecular Dynamics).
Interaction of T125A and T125D mutant proteins with p107 and p130.

In Chapter 2 I have demonstrated the interaction of JCV T' proteins with RB family members which leads to the release of E2F family of transcription factors from the RB-E2F complexes. To determine whether the T125 mutant proteins exhibit these properties, a co-IP/WB approach was taken using extracts of the R2-T125A and R2-T125D cell lines (Figure 3.7). Interactions between JCV proteins and cellular pRb were not detected in these experiments, presumably because of the low levels of pRb in rodent cells (133). Complex formation between JCV early proteins and p107 and p130 was observed in extracts of R2-JCV_E, R2-T125A, R2-T125D and R2-T'_135 cells. In each extract the viral proteins associated with only a fraction of the total pool of p107 or p130; slightly greater amounts of these cellular proteins were consistently found to co-immunoprecipitate with T125D mutant proteins. Further, while the WT and mutant JCV proteins each interacted with only the faster migrating forms of p107 and p130, the total cellular pool of phosphorylated forms of p107 and p130 in the R2-JCV_E, R2-T125A, R2-T125D and R2-T'_135 lines varied. Very small populations of hyperphosphorylated p107 and p130 were detected in R2-JCV_E cells. These modified proteins were detected at higher levels in R2-T125A and R2-T'_135 cells, but were absent in the R2-T125D line. p130 migrates in multiple protein bands due to differential phosphorylation, in my experiments the slowest running form of p130 was affected and not the intermediate forms.
Figure 3.7: Interaction of T125A and T125D mutant proteins with p107 and p130.

Whole cell extracts were prepared from subconfluent R2CT, R2-JCV<sub>E</sub>, R2-T125A, R2-T125D and R2-T<sub>135</sub> cells, and proteins were immunoprecipitated with either PAb 962, anti-p107 or anti-p130 antibody. Co-immunoprecipitated proteins were separated on a 6% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane and probed with either anti-p107 (A) or anti-p130 (B) antibody. Anti-rabbit IgG alkaline phosphatase conjugate antibody was used as the secondary antibody, and protein bands were visualized using NBT/BCIP substrate buffer solution.
The T125D mutant proteins induce the release of transcriptionally-active E2F-1 and E2F-4.

Intact J and LXCXE domains of the JCV early proteins are essential to interact with the RB family members and effect the release of E2F from RB-E2F complexes (Chapter 2). Mutation of the T125 residue altered the interactions between JCV early proteins and p107 and p130 suggesting a role for this residue in the release of E2F-1 and E2F-4 from RB-E2F complexes in a cell. We tested this prediction by conducting luciferase reporter assays in the R2-JCV<sub>E</sub>, R2-T125A and R2-T125D lines. Cells were co-transfected with a β-gal vector (pCMV-βgal) and either an E2F-1 luciferase vector (pE2F1-luc) or E2F-4 luciferase vector (pE2F4-luc), and the activity of the reporter gene was measured. Luciferase activity was normalized based on the β-gal measurements, and the fold activity was expressed relative to activities measured in the parental Rat 2 cells (Figure 3.8). Luciferase activity was nearly undetectable in cells transfected with the promoter-less pΔE2F-Luc construct (data not shown). Induction of luciferase activity in R2-T125D cells transfected with the pE2F1-luc plasmid was consistently greater (7 fold) than that measured in WT R2-JCV<sub>E</sub> cells (5 fold). Transfection of these two lines with the pE2F4-luc vector yielded similar levels of luciferase activity that were reduced relative to that measured in the parental Rat 2 control line. This observation supports the recent finding that E2F-4, a repressive transcription factor, is released from RB-E2F complexes by functional JCV early proteins (Chapter 2). Transfection of R2-T125A cells with either E2F-reporter construct yielded luciferase activities that were similar to those observed in the parental Rat 2 cells.
Figure 3.8: E2F-1 and E2F-4 induced luciferase activity in R2-T125A and R2-T125D cell lines.

Rat 2, R2CT, R2-JCVE, R2-T125A and R2-T125D cells (2.5x10^5) were seeded into 6 well (35 mm) plates. After 12 hrs cells were transfected with 750 ng pE2F1-Luc or pE2F4-Luc and 500 ng pCMV-βgal using Lipofectamine 2000 reagent and incubated at 37°C for 3 hrs. Cells were lysed 48 hrs later and extracts were assayed for luciferase and β-gal activities. The luciferase activity for each sample was normalized using the β-gal readings, and plotted as a fold induction (E2F-1) or fold decrease (E2F-4) relative to the activity measured in the parental Rat 2 cell line (value of 1.0). The E2F-1 and E2F-4 experiments were each repeated 4 times with duplicate samples. The error bars represent the range between duplicate samples in each representative experiment.
**R2-T125D cells are not growth arrested by serum starvation.**

Proliferation of cells in the presence of reduced concentrations of growth factors in the culture medium is correlated with transformation (51). We tested whether our TAg mutant cell lines could be growth arrested under low serum conditions and then progress through the cell cycle upon serum stimulation. The R2-JCV<sub>E</sub>, R2-T125A, R2-T125D, R2-SV40<sub>E</sub>, R2CT and Rat 2 lines were first placed in medium containing 0.01% BCS for 72 hrs, followed by stimulation with medium containing 10% BCS. The progression of synchronized populations of cells through different cell cycle phases (G<sub>0</sub>/G<sub>1</sub>, S and G<sub>2</sub>/M) was analyzed by FACS analysis. Parental Rat 2 and R2CT (G418-selected Rat 2) cells were employed as controls to eliminate differences in cell cycle distribution that might arise from the G418-selection process. FACS analysis showed that following serum deprivation (0 hrs post-serum stimulation), greater than 80% of the Rat 2, R2CT, R2-JCV<sub>E</sub> and R2-T125A cells were arrested in G<sub>0</sub>/G<sub>1</sub> phase, whereas the SV40 control line and R2-T125D cells continued to progress through the cell cycle (Figure 3.9). After serum stimulation the parental cells (Rat 2 and R2CT), R2-JCV<sub>E</sub> and R2-T125A progressed through the different stages of the cell cycle during the course of the experiment, while the pattern of cell cycle progression for SV40<sub>E</sub> and R2-T125D cells at 9, 12 and 15 hrs post-serum stimulation was not altered significantly from that seen at the 0 hr time point.
Figure 3.9: Cell cycle analysis of R2-T125A and R2-T125D mutant cell lines. Rat 2 (R2), R2CT, R2-JCV\textsubscript{E}, R2-T125A, R2-T125D and R2-SV40\textsubscript{E} cells (2.2x10\textsuperscript{5}) were seeded into 6 well (35 mm) plates. After 3 hrs the medium was replaced, and cells were incubated in DMEM containing 0.01% BCS to induce growth arrest. After 72 hrs, cells were refed with DMEM containing 10% BCS and analyzed for cell cycle progression at 0, 9, 12 and 15 hrs post-serum stimulation. The percentage of cells in G\textsubscript{1}, S and G\textsubscript{2}/M phase for each cell line at each time point was determined using an XL-MCL Coulter machine (Center for Quantitative Cell Analysis, Penn State University). The error bars represent the standard deviation between three independent experiments. The data shown above is from a single representative experiment.
Mutation of threonine 125 influences the ability of JCV early proteins to induce anchorage-independent growth of Rat 2 cells.

Cells exhibiting AIG are considered to be transformed. To test whether the mutations at residue 125 altered the ability of JCV tumor proteins to induce a transformed phenotype, we examined the ability of R2-T125A and R2-T125D cells to form colonies in soft agarose (Table 4). Parental Rat 2 cells and M1R2/7 cells, a JCV-transformed Rat 2 line isolated from a dense focus assay (8), were included as negative and positive controls, respectively. The M1R2/7 line, which was originally selected on the basis of its transformed phenotype, readily formed large colonies (10%), whereas G418-selected cells expressing a WT early region (R2-JCV<sub>E</sub>), formed colonies less efficiently (2%). The R2-T125A line, like the parental Rat 2 cells, failed to grow under these conditions, however, R2-T125D cells demonstrated efficient AIG (6%).
Table 4: Anchorage Independent Growth of G418 selected Rat 2 cell lines.

<table>
<thead>
<tr>
<th>CELL LINES&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% CELLS FORMING COLONIES&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat 2</td>
<td>0</td>
</tr>
<tr>
<td>M1 R2/7</td>
<td>10</td>
</tr>
<tr>
<td>R2-JCV&lt;sub&gt;E&lt;/sub&gt;</td>
<td>2</td>
</tr>
<tr>
<td>R2-T125A</td>
<td>0</td>
</tr>
<tr>
<td>R2-T125D</td>
<td>6</td>
</tr>
</tbody>
</table>

<sup>a</sup> AIG of G418 selected Rat 2 cells expressing WT (R2-JCV<sub>E</sub>), T125A mutant (R2-T125A) or T125D mutant (R2-T125D) proteins compared to parental Rat-2 cells and a JCV transformed line (M1 R2/7).

<sup>b</sup> Colonies larger than 0.05 mm were counted in 20 randomly selected fields from 2-60 mm dishes, and the percentage of cells developing into colonies was calculated.
The threonine 125 residue in TAg plays an important role in viral replication.

Previous studies indicated that phosphorylation of T125 and T124 in the JCV and SV40 TAgS, respectively, were critical to viral DNA replication and viability (93, 114). However, the JCV T125D mutant created in the current study was active in a number of assays, perhaps because the negative charge at this position mimicked a phosphorylated T125 residue. Therefore, the DNA replication activity of the T125D mutant virus was compared to that of the T125A mutant and WT JCV. Viral DNAs were transfected into PHFG cells and Hirt-extracted DNA was collected at 0, 10 and 14 days p.t. Relative to WT JCV, both the T125A and T125D mutants were replication defective (Figure 3.10).
Figure 3.10: DNA replication activities of the intact JCV WT and mutant T125A and T125D genomes in human cells.

PHFG cells were seeded into 60 mm plates. After 12 hrs the cells were transfected with JCV, T125A or T125D viral DNA using Lipofectamine 2000 reagent. After 3 hr incubation, medium containing 10% FBS was added to the transfected cells, and at days 0, 10 and 14 p.t., low molecular weight DNAs were collected using the Hirt procedure. The samples shown above are from one of the two independent DpnI replication assays. The bands representing the replicated DNA are indicated (arrow).
3.5 DISCUSSION

Phosphorylation of amino- and carboxy-terminal clusters of serine and threonine residues in the SV40 and JCV TAg s influence multiple functions of these regulatory proteins (28, 91, 114, 115). JCV TAg’s critical role in viral DNA replication has been established, and TAg isoforms (T'135, T'136 and T'165) contribute to replication efficiency (63, 81, 115). Genetic analysis of T'-deficient virus shows a 10 to 20 fold reduction in DNA replication (81, 124). In previous work we demonstrated that JCV TAg T125A mutant is replication incompetent (115). The present study characterizes the phenotypes of JCV tumor proteins mutated at residue 125.

Analysis of cell lines stably expressing T125A or T125D mutant proteins indicates that the T125 residue is essential to TAg stability and early viral protein function. Altered TAg stability could be due to the loss of a phosphorylated residue or due to the substitution of an alanine residue that destabilizes the protein. The substitution of a second amino acid (aspartic acid) for T125 yielded a stable TAg, suggesting that the presence of a negatively charged residue at this position might be required for stability. We investigated the temperature-sensitive phenotype of TAg in R2-T125A cells by incubating cells at 32, 35, 40 and 42°C for 72 hrs prior to conducting IP/WB analysis. TAg expression was not detected in any of the samples (data not shown). In addition, pulse-chase experiments were carried out after pulsing the cells for a short duration (15 and 30 minutes), but the mutant TAg was not detected under these conditions (data not shown).
We attempted to inhibit degradation of the mutant JCV TAg by treating R2-T125A cells with the proteosome inhibitor, MG-132, and in the process we recovered a 42 kD amino-terminal TAg fragment on our gels. However, we were unable to detect the carboxy-terminal fragment with our IP/WB assay. It is possible that degradation of TAg involves multiple cleavages of the protein’s carboxy-terminal half by a cellular protease, followed by ubiquitination and destruction within the proteosome machinery. Activation of the cleavage site(s) would likely result from a structural change in TAg involving either the alanine substitution or loss of the phosphorylated residue at position 125. The detection of all three T’ proteins with the T125A mutation might indicate the mutation either fails to induce structural changes that lead to protease activation or that T’ proteins lack the sequences recognized by the protease.

We observed that T’ proteins expressed in R2-T125A cells migrate as single bands rather than as doublets. T’ proteins typically migrate as hypophosphorylated and hyperphosphorylated bands in proliferating cells; in quiescent cells only a faster migrating form is detected (Bollag et al., in preparation). Our results support the suggestion that the hyperphosphorylated band represents a T’ protein that is modified at T125. Furthermore, these results indicate that modification of this site might be influenced by the stage of the cell cycle through which the cell is passing. This latter suggestion is supported by reports that indicate both the T125 and T124 sites in JCV and SV40 early proteins, respectively, are part of a Cdk consensus sequence and in the case of the SV40, T125 is phosphorylated in vitro by a Cdk (69).

The JCV TAg and T’ proteins contain a functional NLS (amino acids 127-132) immediately adjacent to the T125 site (Bollag et al., manuscript in preparation). A similar
arrangement occurs in the SV40 TAg and 17kT protein, and substitution mutations at the T124 site results in a defect in nuclear import (48). To determine if the T125A and T125D mutations altered TAg localization, immunofluorescence experiments were conducted with the R2-T125A, R2-T125D and R2-JCV_E cell lines. Because the TAg in R2-T125A cells is labile, we examined these cells both before and after treatment with the MG-132 inhibitor. It should be noted that a positive nuclear signal in this assay establishes the presence of TAg only. While T’ proteins do localize to the nucleus, we are only able to visualize this event using EGFP-T’ fusion proteins (Bollag et al., manuscript in preparation). As expected, nuclear TAg staining was readily observed in R2-T125D and R2-JCV_E cells, but not in R2-T125A cells. Treatment of this latter cell line with MG-132 resulted in the appearance of TAg fluorescence, although it is unclear at present whether the atypical staining observed reflects altered nuclear localization of a partially degraded TAg molecule. Our results do indicate that, unlike SV40 TAg, mutation of the T125 site in JCV TAg does not interfere with nuclear import.

We have demonstrated that JCV T’ proteins interact with pRb, p107 and p130 in vitro and in vivo (9; Bollag et al., manuscript in preparation). We have also shown that interactions of JCV TAg and T’ proteins with p107 and p130 require an LXCXE motif (residues 105-109 of the viral proteins), and that degradation and/or dephosphorylation of hyperphosphorylated p107 and p130 by the viral proteins require a J domain positioned within the first TAg/T’ exon (Chapter 2). The present observations confirm that the T125 site also influences the ability of JCV early proteins to bind and to alter the cellular pools of RB proteins. Relative to the JCV WT proteins, T125A mutant proteins bind p107 and p130, and reduce the levels of RB hyperphosphorylated species with slightly reduced
efficiency. On the other hand, these two activities are enhanced when measured in R2-T125D cells. We cannot rule out the possibility that reduced binding of RB proteins in the R2-T125A cells is due to the absence of a stable TAg. However, we have shown that binding of p107 and p130 in vivo is primarily a function of JCV T' proteins, not TAg (Figure 3.6; Bollag et al., manuscript in preparation). Thus, we speculate that the slight reduction in RB binding exhibited by the mutant T' proteins is due to the T125A mutation and not due to the loss of TAg. These observations lead us to suggest that only a subset of differentially-modified early JCV proteins regulate the RB pathway. Relevant to our hypothesis, Sullivan et al. (111) have reported that only a small fraction of SV40 TAg binds to RB proteins in cells.

Recently we demonstrated that JCV T' proteins effect the release of E2F-1 and E2F-4 from RB-E2F complexes in rat fibroblasts (Chapter 2). To test the prediction that differential binding of JCV WT and mutant proteins to p107 and p130 results in differential regulation of E2F-1 and E2F-4 members, we measured free, transcriptionally-active E2F in R2-JCV_E, R2-T125A and R2-T125D cells. In agreement with our binding data, we measured minimal effects on E2F-1 and E2F-4 activation in R2-T125A cells, but detected consistently higher levels of E2F release in R2-T125D cells. It appears that the T125A mutation had a greater negative impact on the activation of E2F and the disappearance of hyperphosphorylated RB species than it did on the binding of p107 and p130.

The ability of JCV early proteins to effect the release of transcriptionally-active E2F from E2F-RB complexes is expected to have significant consequences for cell growth. To investigate the impact of E2F release on cell cycle progression, cells
expressing JCV WT or T125 mutant proteins were serum starved for 72 hrs, refed with medium supplemented with 10% BCS and then subjected to FACS analysis at several times post-serum stimulation. A high percentage of R2-T125D and R2-SV40_E (Rat 2 cells expressing an intact SV40 early region) cells were distributed throughout S phase at the 0 hr time point, indicating these cells were not growth arrested even under stringent low serum conditions. The remaining lines, including R2-JCV_E, R2-T125A and two lines of Rat 2 cells (parental Rat 2 and G418-selected Rat 2 cells [R2CT]) were growth arrested when incubated in 0.01% serum. By the 9 hr time point, R2-JCV_E and R2-T125A cells had entered S phase. The two Rat 2 control lines were the last to leave G0/G1 and the R2CT cells appeared to enter S phase slightly ahead of the parental Rat 2 line. These data suggest a possible correlation between cell cycle progression and the patterns of E2F-1 and E2F-4 release promoted by the WT and mutant JCV tumor proteins (Figure 3.7), however, it will be necessary to look at additional cloned lines to confirm these results.

We believe that our failure to growth arrest R2-T125D cells in medium containing low serum concentrations reveals an important difference in the abilities of JCV WT and T125D tumor proteins to influence cellular proliferation. Growth in low serum is one property exhibited by virally-transformed cells. An even more stringent parameter associated with polyomavirus transformation of mammalian cells is the ability of those cells to exhibit anchorage-independent growth (AIG). Induction of AIG in rodent cells by SV40 TAg requires an intact RB-binding domain, but it is unclear whether a functional J domain is also required (109, 118). We find that cells expressing the JCV T125D proteins grew more efficiently in soft agarose than did cells expressing the JCV WT proteins.
Thus, in addition to demonstrating greater effects on RB proteins function, the T125D mutant proteins exhibited a more robust effect on this transformation parameter than did the WT early proteins. On the other hand, cells producing the T125A proteins failed to exhibit AIG. We have observed that JCV constructs expressing a subset of the early proteins fail to induce transformation as measured by a dense focus assay (Kilpatrick and Frisque, unpublished data). Because TAg in R2-T125A cells is unstable, we predicted that these cells would not grow in soft agarose.

Phosphorylation of the T124 site in SV40 TAg is required for efficient viral DNA replication (93). Kim and co-workers (50) proposed that during replication initiation, TAg utilizes an unphosphorylated CDK/NLS motif to bind the core replication origin and form the first of two hexamer structures. The CDK consensus signal in this motif includes the T124 residue. Once T124 phosphorylation occurs in a cell cycle dependent manner, the second hexamer is formed, creating the TAg helicase required for the elongation step of replication. The failure of both the T125A and T125D JCV mutants to replicate in human cells is consistent with the predictions of this model. As suggested by the model, some functions of TAg and TAg isoforms require that a specific residue alternate between a phosphorylated and unphosphorylated state. In the present study, mutant JCV TAgS, T125D and T125A, represent the continuously phosphorylated or dephosphorylated states, respectively. These two mutant TAgS, unlike WT proteins, are unable to switch to the alternate form, thus rendering both mutant viruses replication defective. In contrast, JCV TAg and T’ proteins may carry out some functions only when residue 125 is post-translationally modified. In the current study, we found that T125D proteins, which represent constitutively “phosphorylated” forms, interacted with RB-E2F
complexes and transformed cells more efficiently than did WT JCV proteins that exist as a mixture of phosphorylated and unphosphorylated species.

The polyomavirus TAg s are phosphorylated at multiple sites, in several combinations and at different times during the cell cycle. With the discovery of TAg isoforms, an even greater number of tumor protein subpopulations are now known to exist in infected and transformed cells. The multitude of interactions occurring between these viral proteins and the cellular factors that drive the replication and cell cycle machinery most certainly contribute to the regulatory complexities involved in virus-host interactions.
Oncoproteins encoded by DNA tumor viruses have evolved to perturb host cell regulatory circuits in ways that favor viral replication. In uninfected cells these same circuits regulate cell proliferation and survival. The SV40 TAg, adenovirus E1A and E1B and human papillomavirus E6 and E7 proteins are among the most thoroughly characterized viral tumor proteins. These oncoproteins share the ability to bind and inactivate the pRB and p53 tumor suppressor proteins (26, 57, 74), two cellular proteins that regulate important pathways often disrupted in human cancers (94).

**Cellular Transformation by viral oncoproteins.**

Expression of oncoproteins in nonpermissive cells may lead to transformation, a process *in vitro* that serves as a model for *in vivo* tumorigenicity. For successful transformation usually two oncoproteins are needed, one to immortalize the cells and the other to complete the transformed phenotype. Under some conditions SV40 TAg alone has the ability to immortalize and transform cells. Different domains have been mapped on SV40 TAg that are involved in the transformation process. The N-terminus of TAg includes two motifs, the J and LXCXE domains that contribute to transformation of human and rodent cells. Analysis of J domain mutants in rodent cells showed that this domain was found to be required to induce (i) growth to high density, (ii) growth under low serum conditions, and (iii) immortalization. Studies using human cells showed that the J domain was dispensable for immortalization or AIG properties. Similarly, analysis of LXCXE mutants revealed that these sequences influence immortalization of rodent cells.
SV40 tAg also plays an essential role in the transformation of both rodent and human cells under some conditions. In rodent cells, under conditions when TAg expression is low, tAg expression is necessary for transformation. In addition, tAg expression is necessary to transform rodent cells that are quiescent. SV40 mutants defective in tAg production are unable to transform human fibroblasts, but transform murine fibroblasts with reduced efficiency (21). In human mesothelial cells, both TAg and tAg expression transform cells more readily on exposure to carcinogens than cells expressing TAg alone (7). In rodent cells, although expression of SV40 TAg alone is sufficient to induce transformation, coexpression of TAg and an oncogenic allele of \textit{H-ras} gene enhances transformation to tumorigenicity (44, 70). However, expression of these two oncogenes in human cells leads to irreversible growth arrest or apoptosis, instead of transformation and tumorigenicity. The introduction of the gene encoding the catalytic subunit of human telomerase (hTERT) along with SV40 early region immortalizes human fibroblasts, human embryonic kidney (HEK) cells and human mammary epithelial cells (HMEC), and these cells acquire a tumorigenic phenotype upon subsequent introduction of the \textit{H-ras} oncogene (40) and references therein). Telomerase is constitutively expressed in rodent cells, but is inactive in normal human cells. This difference provides one explanation for species-specific differences in virally induced transformation.

Even though human cells expressing TAg, hTERT and Ras are immortal, their doubling time is three times faster with the expression of tAg. tAg activates PI3K, an inducer of antiapoptotic signaling cascade (137). Considering Ras can also activate the PI3K cascade, it is possible that a high survival response is required in human cells for
efficient transformation. In addition to the activation of PI3K, tAg interacts and sequesters the protein phosphatase PP2A. Mutants of tAg that fail to interact with PP2A also fail to transform cells (40). PP2A represents a large and complex family of enzymes and combinatorial association of its various subunits regulate hundreds of distinct cellular phosphoprotein substrates, making it difficult to predict the precise biochemical alteration due to the tAg interaction.

Compared to SV40, only a limited number of studies have investigated the oncogenic potential of JCV TAg, tAg and the three T’ proteins. An intact JCV early region induces dense focus formation in Rat 2 cells only inefficiently (8). Individual JCV early proteins expressed from a CMV promoter fail to transform these cells. However, isolation of Rat 2 cells expressing JCV tumor proteins by G418 selection revealed that the cells exhibited some minimally-transformed growth parameters (123), Bollag et al., in preparation). To investigate the contribution of individual T’ proteins in viral transforming behavior, a Ras cooperation assay was performed recently (Frisque, unpublished data). In this assay both T’_{135} and T’_{136} overcame Ras-induced cell growth arrest and led to immortalization of primary cells; about 50% of the cell clones expressing T’_{165} became immortalized. TAg, on the other hand, immortalized only 2 out of the 18 cell lines tested. The ability of JCV T’ proteins and not TAg to immortalize rodent cells and overcome Ras-induced cell cycle arrest suggests that these proteins influence JCV biology via different mechanisms.
Regulation of RB/E2F pathway by the viral oncogenes.

Interaction of viral oncoproteins with RB family members creates an S phase environment in cells for viral replication, although the precise mechanism behind this process is still being unraveled. Biochemical studies and in vivo analysis of gene promoters using adenovirus E1A protein reveal two significant findings. First E1A activates a number of repressed E2F-targeted genes that are involved in DNA replication. Second, E1A mediates this repression by perturbing occupants of the E2F promoters. In quiescent or growth-arrested cells p130/E2F-4 complexes recruit histone deacetylase (HDAC) components to promoter sites and associate with the methylase SUV39H, leading to the methylation of the histone H3 tails at these promoter sites. Interaction of E1A with p130/E2F-4 complexes leads to the release of p130 from the promoters, which in turn leads to the release of SUV39H from these promoters. Once the promoter is free of repressive complexes the surrounding nucleosomal histones become acetylated. E1A-mediated removal of methyl groups from histone H3 not only allows for the acetylation of Lys-9, Lys-14, but also the phosphorylation of Ser-10. These changes in the chromatin structures are correlated with transcriptional activation in both yeast and mammals (125). The removal of p130/E2F-4 from the promoters and the acetylation of histone H3 coincide with the recruitment of pRb/E2F-1 to the E2F target gene promoters. It is not known if other types of histone modifications or ATP dependent chromatin remodeling complexes such as SWI/SNF are involved in this regulation (36).

The ability of JCV early proteins to promote a cell’s entry into S phase was examined by determining the effects of TAg and the three T’ proteins on repressor (p130/E2F-4), and activator (pRb/E2F-1) complexes. The studies described in Chapter 2
show that different T’ proteins have different effects on repressor and activator RB/E2F complexes. These studies however do not address the effects of RB/E2F regulation on histone tail modifications. Further, the observations suggested that progression of cells from G₀/G₁ into S phase of cell cycle is mainly due to the coordinated action of all the JCV early proteins, rather than action of a single T’ protein. Although, our studies did not address tAg’s role in S phase progression, one can speculate that tAg regulates the cell survival and proliferation signals via PP2A interaction, ultimately contributing towards S phase progression.

**Role of J and LXCXE domains in RB/E2F regulation.**

Biochemical studies suggest that the interaction of JCV TAg and T’ proteins with RB members and the release of free E2F involve multiple steps. The J and LXCXE domain mutants have helped to dissect some of the functions of these two domains in RB interaction and E2F release. Analysis of an LXCXE domain mutant shows that (i) this domain is essential to the interactions between viral proteins and RB family members and (ii) the turnover of hyperphosphorylated p130 and p107 likely requires a physical interaction between the viral oncoproteins and p130 and p107. Characterization of the J domain mutant has revealed that (i) the HPD motif of this J domain plays a role in the turnover of hyperphosphorylated p130 and p107 and (ii) in the presence of a nonfunctional J domain, viral early proteins interact with both the hyper- and hypophosphorylated forms of p107 and p130. Interestingly, Brown and Gallie (11) observed that mutation of the pRb B-domain lysine patch to arginines promotes TAg binding to both the hypo- and hyperphosphorylated forms of pRb, similar to my J domain
mutant H42Q. The H42Q mutant also exhibits leakiness in other assays, such as E2F release and cell cycle progression, suggesting other regions of TAg might compensate for this J domain mutation. Differences in SV40 transforming activity are observed based on the TAg context in which the J domain mutation is introduced. In a full length TAg, the presence of a D44N mutation has only a small effect on transforming efficiency whereas in an N-terminal truncated TAg (T136) the mutation has a much greater effect. Further, the D44N mutant TAg forms a stable complex with Hsc70, but the T136 D44N mutant TAg only forms a transient complex (29). Additional observations argue that mutation of lysine 53 in SV40 TAg (K53R), a solvent-exposed residue oriented opposite the hsc70-interacting face, does not form a stabilizing interhelix contact with Hsc70, and has an impaired chaperone function. Thus, sequences outside the HPD motif might contribute to the chaperone activity of the J domain.

Recently, Nemathova et al., (75) suggested a two step mechanism for E2F transactivation by mPyV large T (TAg). The authors propose that TAg interacts with the RB-E2F complexes via its N-terminal LXCXE and J domains to cause the release of E2F, subsequently the C-terminus of TAg recruits co-activators p300/CBP-PCAF, which results in activation of E2F-responsive promoters. Based on this two step model for mPyV TAg, JCV TAg, which has a C-terminal p300/CBP-PCAF binding sequence, should activate E2F promoters, while T' proteins that lack this sequence should exhibit reduced or no activation of E2F promoters. The data described in Chapter 2 contradicts this two step model, further suggesting that JCV TAg could be regulated via a novel mechanism in cells.
Does TAg induce apoptosis in cells?

The cellular response to unscheduled proliferation is to induce apoptosis, and viruses in turn have evolved ways to block cellular apoptotic signals to promote efficient viral replication. Past attempts to create stable cell lines expressing JCV TAg have been unsuccessful (Bollag et al., in preparation). This observation is surprising considering TAg can bind and inactivate p53 in cells. Inactivated p53 suppresses induction of the downstream cyclin-dependent kinase inhibitor p21 that promotes arrest of cells in the G1 phase of the cell cycle. In addition to cell cycle arrest, p53 can induce apoptosis via an intrinsic pathway. It should be noted that SV40 TAg and tAg may induce apoptosis in certain cell lines, and under certain conditions. Thus, our inability to obtain TAg expressing Rat 2 and 3T3 cell lines suggested that JCV TAg could induce apoptosis in these rodent cells. Because cell lines expressing individual T' proteins and all five JCV early proteins were derived readily, it is possible that tAg and/or T' proteins block TAg-induced apoptosis in cells. However, initial attempts to confirm JCV TAg-induced apoptosis in lines of Rat (Rat 2) or mouse (NIH-3T3) fibroblasts have been unsuccessful.

Biochemical studies have demonstrated that TAg interacts with all three RB family members in vitro. The in vivo study described in Chapter 2 demonstrates the inability of TAg to release free E2F from the RB/E2F complexes. Recently, pRb has been shown to exert antiapoptotic effect in both human and rodent cells by binding to promoters of apoptotic genes and repressing their expression (133). It has been observed that only a small subset of JCV and SV40 TAg interacts with pRb both in vitro and in vivo (9, 111). It is possible that a subset TAg interacts with the repressive pRb/E2F
complexes on apoptotic gene promoters and effectively releases pRb from these promoters thereby inducing apoptosis.

**Regulation of JCV early proteins by post-translational modifications.**

Protein phosphorylation/dephosphorylation is a key regulatory mechanism in eukaryotic cells that is targeted by oncogene and proto-oncogene products to influence cellular growth control (reviewed by (46). Cell cycle progression is dependent on phosphorylation and dephosphorylation of key regulators including RB and E2F family members and p53. Studies described in Chapters 2 and 3 shows the interaction of JCV early proteins with p107 and p130 is stronger when phosphorylated. The phosphorylation status of cellular proteins in turn is known to affect their association with viral oncoproteins. Phosphorylation of Thr 821 and 826 sites on pRb releases SV40 TAg from the complex (52). Structural analyses suggest that phosphorylated C-terminal polypeptide of pRB competes for the same binding site as an LXCXE peptide, and displaces the peptide. This displacement is aided by a six-lysine basic patch on the rim of the LXCXE binding site (55). Furthermore, this B-domain lysine patch is required for pRb binding to SV40 TAg and E2F release (11).

The importance of Thr125 residue phosphorylation in JCV early proteins has been discussed in Chapter 3. Replacement of this threonine with an alanine residue prevents modification of this site, whereas substitution with an aspartic acid would mimic the negative charge of a phosphate group and prevent reversion to an unphosphorylated state. In infected cells, several subpopulations of TAg are present based upon their
phosphorylation patterns. This heterogeneity makes it difficult to address the contributions of individual and multiple phosphorylation sites on protein function.

Analysis of T125A and T125D cell lines show that the retardation of the T' protein migration on an SDS gel is primarily due to Thr 125 phosphorylation. In addition to contributing to protein migration differences, phosphorylation of this residue impairs the ability of T/T' proteins to interact with RB family members, release E2F and promote cell cycle regulation. On the other hand, T125D mutant early proteins bind RB members and release E2F more efficiently than WT proteins. Further, T125D cells cannot be growth arrested by serum starvation, and these cells readily exhibit an anchorage-independent growth. These observations suggest that in addition to the J and LXCXE domains, phosphorylation of the T125 residue is required to effectively release E2F from the RB/E2F complexes. Even though functions of the T125D mutant are enhanced in some assays, JCV (T125D) is replication-defective. Given that both T125A and T125D mutant viruses fail to replicate, it appears that switching between modified states is essential for certain protein functions. The roles of phosphorylated and unphosphorylated TAg in SV40 replication, nuclear import and cell cycle progression are well characterized (48), while similar studies in JCV has been complicated by TAg instability in T125A cells. Elucidating the molecular mechanism behind TAg instability in T125A cells might pave the way for understanding the role of T125 phosphorylation in JCV TAg.
Protein degradation machinery in eukaryotes nucleus.

The instability of the T125A TAg and our inability to rescue the C-terminal TAg fragment(s) with proteosome inhibitor MG-132 treatment is intriguing. Furthermore, the N-terminal 42 kDa TAg fragment which is rescued by MG-132 treatment is targeted to the nucleus like full length WT TAg. However, the nuclear staining pattern of the 42 kDa TAg fragment differs from that of full length TAg. The 42 kDa fragment exhibits an unusual punctate staining which may denote aggregated protein localized to protein degradation sites within the nucleus.

Aberrant proteins arise in cells, in part, because of mutation or transcriptional or translational errors. The protein quality control system within the cell marks such proteins for degradation, usually via the ubiquitination system (108). It is estimated that a third of newly synthesized proteins are defective and are destroyed during or shortly after synthesis. Because there is no functional protein synthesis machinery in the nucleus, it is still unclear how a cell contends with aberrant proteins in this cellular compartment. A recent report identified a protein quality control system within the nucleus of *Saccharomyces cerevisiae* (34). San1p, a ubiquitin ligase, is the defining member of this degradation system. In this study San1p was found to differentiate between mutant and wild type nuclear proteins, sending the mutant for rapid degradation.

N- or C-terminal phosphorylation of SV40 TAg is influenced by phosphorylation of residues at the other end of the molecule, suggesting that an interaction between SV40 TAg N- and C-terminal residues occurs (91). The same arrangement of two terminal phosphorylation domains in the JCV TAg suggest that a phosphorylation defect at the threonine 125 residue might play a role in altering interactions with the TAg C-terminal
residue(s), resulting in structure destabilization and degradation via the proteosome machinery. Further more, instabilities could occur by other mechanisms as described in Chapter 3.

**Concluding remarks.**

The overall goal of this thesis was to elucidate the role of JCV TAg and T' proteins in E2F regulation and cell cycle progression. The studies described in Chapter 2 suggest that while JCV TAg is not effective in RB regulation and E2F release, its isoforms, the T' proteins, efficiently release E2F-1 and E2F-4 from RB/E2F complexes. As a multifunctional protein, TAg must regulate numerous cellular proteins to aid viral replication, perhaps leaving the T' proteins to regulate RB members to promote cell cycle progression (Figure 2.17). My findings also indicate that progression of cells from G_o/G_1 into S phase and efficient transformation of Rat 2 cells requires expression of the intact early region. The contributions of the LXCXE and J domains mutants to RB binding, E2F regulation and viral replication have been elucidated by the analysis of relevant mutants. However, the role of the J domain in AIG and E2F release could not be ascertained due to the leaky phenotype of the H42Q mutant. Even though T' proteins share their J domain with TAg, the role of individual J domain in RB regulation and E2F release is not addressed here. It is likely that some or all three T' proteins complement some of the TAg J domain functions under certain growth conditions or in specific cell types. SV40 17 KT has been shown to complement DnaJ mutations in TAg and restore transformation of primary human fibroblast (10). Studies described in Chapter 3 show that while the T125A mutation yields an unstable TAg, the T125D mutation enhances some of the functions of
the viral early proteins. This latter observation may reflect the requirement for a single
constitutively-on phosphorylation state that induces certain cellular alterations such as a
more transformed phenotype (e.g. reduced growth arrest in low serum conditions,
enhanced AIG). Alternatively, the replication defect in T125A and T125D mutant viruses
suggests that some early protein functions require an on/off phosphorylation switch.

These studies make two major observations- (i) JCV TAg differs from other
polyomavirus TAg isoforms. Only now beginning to gain acceptance in
polyomavirus field that TAg isoforms contribute to the complex mechanisms
regulating the virus host interaction.
Chapter 5

FUTURE EXPERIMENTS
To study the role of T’ proteins in chromatin modification:

**Hypothesis:** I hypothesize that disruption of the E2F-4/p130 repressive complex by JCV T’ proteins leads to chromatin modification at the E2F-responsive promoters. Expression of JCV T’ proteins results in a switch of H3 histone from a methylated to an acetylated state, thereby leading to the activation of genes involved in S phase progression.

**Experimental Design:** The “Tet-on” inducible system is helpful in expressing proteins for a controlled duration of time. Induction of protein expression at a desired time facilitates the monitoring of changes in cell morphology that is induced by that protein. By employing tetracycline-inducible cells that express individual T' proteins or combinations of any two T’ proteins, one could analyze histone tail modifications to determine if JCV early proteins influence chromatin modification. Flow cytometric analysis of growth-arrested cells would permit identification of conditions required to synchronize cells in G₀/G₁. RT-PCR analysis of an S phase gene such as cyclin A or cdc6 would confirm a lack of promoter activity in this gene during quiescence.

Methylation of lysine-9 on histone H3 is associated with silencing of genes in regions other than heterochromatin. By crosslinking cells before extract preparation, protein-DNA complexes at promoter regions could be preserved. A chromatin immunoprecipitation (ChIP) assay could be performed using the lysate of crosslinked cells to identify the lys-9 methylation or acetylation state in cyclin A or cdc6 promoter. The ChIP assay include an immunoprecipitation step using lys-9 methylation or lys-9 acetylation specific antibodies, followed by a PCR step using gene specific primers (130).
Analysis of histone tail modifications after expressing individual T’ proteins will provide information on how RB/E2F regulation by individual T’ proteins affects histone modification. Based on the results described in Chapter 2 one would expect T’$_{135}$ expression to demethylate the histones, while T’$_{165}$ expression would be expected to cause histone acetylation.

To study the role of Thr 125 residue in TAg stability.

**Hypothesis:** I hypothesize that the N-terminal threonine 125 phosphorylation stabilizes TAg either by facilitating interaction with another cellular protein or by preventing exposure of protease cleavage sites in TAg which, in unphosphorylated protein, are recognized by cellular proteases.

**Experiment:** TAg containing the T125A mutation is unstable while the T’ proteins which contain the same alteration are stable. Analysis of TAg instability in a stable T125A cell line indicates that the mutant TAg is degraded by the 26S proteosome. Treatment of these cells with the proteosome inhibitor MG-132 rescues the N-terminal 42 kDa fragment of TAg, but the C-terminal fragment(s) is not detected by an IP/WB assay, suggesting proteolytic cleavage of the latter fragment by cellular proteases. Given that T’ proteins and TAg share their first 132 amino acids and the stability of T’ proteins is not affected, it is likely that sequences unique to TAg contains elements that influence protein stability. It is possible that sequences unique to TAg could be interacting with the Thr 125
phosphorylated residue, thus stabilizing the protein. Disruption of Thr 125 phosphorylation in turn could affect TAg folding, thus marking it for degradation.

To investigate these observations, one approach would be to make series of C-terminal deletions in TAg and monitor protein stability. If restriction sites are not available at convenient locations of the gene, one could use a PRC based approach to generate precise deletions. T'\textsubscript{165} shares sequence from nucleotides 2705 to 2603 with TAg in its C-terminus, and considering it is stable in T125A mutant cell line, it is likely that sequence between nucleotides 4274 and 2705 in TAg influences instability. Hence, one could try making deletions in this region of the TAg.

During my attempt to identify TAg C-terminal fragment(s) rescued by MG-132 treatment, I used antibody PAb 901 directed against the sequence located at the extreme C-terminal end of the molecule. It is possible that the C-terminus of TAg is clipped into multiple fragments or at the end of the molecule recognized by PAb 901. In order to look into this possibility, one can try and introduce epitope tags at different locations within the TAg C-terminus and use IP/WB approach to identify fragment(s). A FLAG or an HA tag can be introduced before nucleotide 2705 in TAg and after treatment of cells with MG-132, using a IP/Western blot assay one could probe for the C-terminal fragment(s).

The T' proteins are stable in T125A mutant cells and they share the first 132 amino acids (~nucleotide 4274). It is likely that ubiquitination occurs after this amino acid. The ubiquitination could occur at lysine residue(s) in the N-terminus of the molecule between protein regions ~16 kDa and 42 kDa. The lysine residue(s) beyond 42 kDa protein region could be ubiquitinated and degraded once TAg is cleaved in its C-terminus. Search for protease consensus site near the 42 kDa region of TAg has not
provided any clue for any potential proteases acting on TAg at this site. If one identifies potential protease(s), then one might be able to use specific protease inhibitors to test their effect on TAg. In a second approach one could create a truncated TAg composed of the N-terminal 42 kDa of the protein, similar to the TAg fragment detected after treatment with proteosome inhibitor MG-132. Expression of N-terminal fragment of TAg by itself and looking for stability will provide clue on the influence of C-terminal sequence for N-terminal stability. Further, T136, a truncated SV40 TAg expressing only the N-terminal 136 amino acids is stable in cells. Therefore instability of JCV TAg N-terminal fragment in cells would suggest presence of ubiquitination site between nucleotides 4274 and the end of the fragment. T’ proteins share till nucleotide 4274.

In a third approach, one could reexamine TAg stability in a transient transfection and/or in human cell lines, since T125A mutant was found to be unstable in G418-selected Rat 2 cell lines. It is possible that expression of T125A mutant early proteins in a human cell line (e.g. U87MG cells) will make a stable TAg and therefore would be detected by WB. This will help identifying if TAg instability is a cell specific or a mutational effect.
BIBLIOGRAPHY


The experiments described in Chapters 2 and 3 were conducted with Rat 2 cell lines, which are non-permissive for JCV replication. It has been shown that JCV enters both permissive and nonpermissive cells and induces early gene transcription, but the virus replicates, expresses late genes and assembles virions only in the former cells. We have demonstrated earlier that purified JCV TAg and T′ proteins interact with RB family members in human MOLT-4 cells (Bollag et al., 2000). To determine if the results reported in Chapter 2 were reproduced in permissive human cells, I performed E2F luciferase assay in U87MG cells. Since human cell lines stably expressing individual JCV early proteins were not available, U87MG cells were transiently co-transfected with vectors expressing individual or all five JCV early proteins, E2F1-Luc or E2F4-Luc and CMV-βgal. The effect that individual TAg/T′ proteins had on RB/E2F complexes and the release of free E2F was measured by the luciferase reporter assay (Figure A.1). Greater than 7 fold induction in E2F-1 activity was measured consistently in cells expressing all five JCV early proteins. The three T′ proteins exhibited a 2.6-3.5 fold increase in activity while, TAg and the E109K, H42Q and H42Q/D44N mutants had 2 fold or less activity.
Similar analysis of E2F-4 activity in U87MG cells also exhibited differences with observations made in Rat 2 cells (Figure A.2). Cells expressing all five JCV early proteins had no effect on E2F-4 release, while $T'_{135}$ and $T'_{136}$, $T'_{165}$ and TAg had a modest effect; the J and LXCXE domain mutants showed no activity. The J domain double mutant in this particular experiment showed activity for E2F-4 release. This could be explained by the difference in the transfection efficiency between the duplicate samples.
The differences observed in the luciferase activity between Rat 2 and U87MG cells were unexpected. I speculated that differences in viral protein expression in the two cell lines resulted in differences in the luciferase readings. From the studies described in Chapter 2, I had shown that differences in viral protein expression influenced the reporter reading. Hence, I examined viral protein expression levels in U87MG cells and compared them to that in Rat 2 cells. The U87MG and Rat 2 cells were transiently transfected with 500ng

Figure A.2: E2F-4 repressed luciferase activity in transiently transfected U87MG cells.

U87MG cells (2.5x10^5) were seeded in 6 well (35 mm) plates. After 12 hrs the cells were co-transfected with 500 ng of vectors expressing JCV early proteins along with 750 ng pE2F4-Luc and 500 ng CMV-βgal using Lipofectamine 2000 reagent. The experiment was repeated once in duplicate. The error bars represent the standard deviation between duplicate samples in this representative experiment.
CMV-JCV\textsubscript{E} or CMV-T'\textsubscript{165} constructs as these expression vectors exhibited the maximum difference in E2F-1 fold induction between Rat 2 and U87MG cells. To facilitate identifying the protein bands, the cells were labeled in medium containing $^{35}$S-methionine 36 hrs post-transfection. Similar to the luciferase assay, cell lysates were collected 48 hrs post-transfection, and analyzed by IP (Figure A.3). The transfection efficiency in Rat 2 cells was too low to observe distinct JCV protein bands. In U87MG cells expressing JCV early proteins, the TAg, T'\textsubscript{135} and T'\textsubscript{136} protein bands, but not the T'\textsubscript{165} band, were detected. The latter observation is interesting since U87MG cells transfected with CMV-T'\textsubscript{165} does exhibit protein expression. It is possible that splicing factors are present in different ratios and/or act differently in processing the precursor JCV\textsubscript{E} mRNA in U87MG cells compared to Rat 2 cells. CMV-JCV\textsubscript{E} doesn’t express late genes relative to a “natural” infection or transfection of the entire viral genome. Hence, another possibility is that late protein expression influences splicing of the T'\textsubscript{165} transcript. If indeed late viral sequences are necessary for T'\textsubscript{165} expression, then one would expect the expression of T'\textsubscript{165} band in U87MG cells transfected with JCV (Mad 1) DNA.

YB-1, a Y-box nucleic acid binding protein involved in a wide range of gene expression regulation at transcription level, translation level and DNA repair. Stickeler et al., (2001) demonstrated that YB-1 can also act as a splicing factor. It was shown to bind A/C rich exon enhancer and stimulate splicing of human CD44 gene. The JCV late protein LP1 or agnoprotein is known to interact with the splicing factor YB-1. Further, YB- as a transcription factor can activate JCV late promoter.

My attempts to test for the presence of T'\textsubscript{165} expression in the presence of JCV late protein expression did not yield clear results due to high background bands. The role of
agnoprotein in process could be investigated if the late protein expression does influence $T'_{165}$ expression in permissive human cells.

Figure A.3: Protein expression analysis in transiently transfected U87MG cells.

U87MG cells were transiently transfected with 500 ng of CMV-JCV$_E$ or CMV-$T'_{165}$ vectors. The cells were labeled in medium containing $^{35}$S-methionine 36 hrs post-transfection and lysates were collected 48 hrs post-transfection. The protein bands were analyzed by IP.
APPENDIX B

Portions of this thesis, including text and figures were from the following publications:

- **Shiva K. Tyagarajan and Richard J. Frisque** (2005), Phosphorylation of JC virus tumor proteins influences their interaction with p107 and p130 and the subsequent release of E2F (Submitted).

- **Shiva K Tyagarajan and Richard J. Frisque** (2005), T’ proteins contribute to JC virus induced cell cycle progression (Submitted).


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. **Shiva K Tyagarajan** and Richard J. Frisque (2005), T’ proteins contribute to JC virus
induced cell cycle progression (in revision).

. Brigitte Bollag, Lisa Kilpatrick, **Shiva K. Tyagarajan** and Richard J. Frisque (2005),
JCV T’ proteins interact with p107 and p130 *in vivo* and promote transformation of Rat
fibroblast cells (in revision).

. Richard J Frisque, Catherine Hofstetter and **Shiva K. Tyagarajan** Transforming
Activities of JC Virus Early Proteins- Book Chapter (Polyomavirus and Human

. Richard J Frisque, Brigitte Bollag, Shiva K Tyagarajan, and Lisa Kilpatrick (2003), T’