JC VIRUS SMALL T ANTIGEN BINDS RB FAMILY PROTEINS AND THE PHOSPHATASE PP2A AND IS REQUIRED FOR EFFICIENT VIRAL DNA REPLICATION ACTIVITY

A Dissertation in Biochemistry, Microbiology and Molecular Biology by Catherine A. Hofstetter

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ABSTRACT

JC Virus (JCV) is a human polyomavirus which infects approximately 80% of the human population. Five tumor antigen or T proteins are produced from the early coding region of the viral genome; one precursor early mRNA is spliced to generate each of the five mature T protein mRNAs, which encode large Tumor Antigen (TAg), small tumor antigen (tAg), and the three T' proteins, T'\textsubscript{135}, T'\textsubscript{136} and T'\textsubscript{165}. TAg, the best studied of these, is the key viral protein in facilitating DNA replication and cellular manipulation leading to oncogenic transformation. TAg is capable of binding to many diverse cellular factors, including members of the Retinoblastoma family of tumor suppressor proteins and Hsc70 through its LXCXE and J domains, respectively, to influence cellular proliferation, as well as binding and inactivating p53 to circumvent apoptosis. The 3 T' proteins play roles in DNA replication and transformation; despite a high degree of sequence similarity to each other, they interact differentially with cellular proteins to modulate cellular proliferation and viral DNA replication. tAg is suspected to be important in host cell proliferation based on studies performed on the related SV40 tAg.

In this study, I have generated a collection of DNA constructs which express different combinations of the JCV early proteins. These DNAs were used to analyze the effects of each combination of viral proteins on viral DNA replication and modulation of cellular signaling pathways. In particular, tAg’s specific interactions with cellular proteins and its potential role in pathways affecting cellular proliferation were studied. To examine whether specific conserved amino acid residues contributed to tAg’s functions, I constructed a series of DNAs encoding mutant tAg proteins. To create the first mutant tAg, a conserved proline residue at nucleotide 99 was altered to alanine (P99A); in the
second mutant, a conserved cysteine 157, part of tAg’s second LxCxE domain, was changed to alanine (C157A); in the third tAg mutant, histidine 42 in the J-domain was altered to glutamine (H42Q).

TAg was found to contribute to viral DNA replication. In contrast to studies which indicate that SV40 tAg is not required for replication of SV40 DNA, I observed no replication of JCV DNA in cells transfected with constructs that did not express tAg. Viral DNA constructs encoding mutant versions P99A or C157A of tAg exhibited decreased replication activity in permissive primary human fetal glial (PHFG) cells relative to a construct expressing wild type tAg. The defective phenotype was more pronounced in the P99A mutant. These data suggest that residues 99 and 157 of tAg facilitate tAg’s contributions to viral DNA replication.

I have extended a recently published finding that a tAg-GST fusion protein interacts in vitro with protein phosphatase 2A (PP2A), a ubiquitous cellular protein responsible for dephosphorylation and regulation of many substrates. To determine whether this interaction takes place in vivo, I tested the ability of wild type tAg as well as the P99A and C157A mutant tAgs described above to interact with PP2A. Neither of these point mutations in tAg led to a detectable decrease in PP2A binding. Preliminary data suggests that expression of tAg increases the amount of phosphorylated Akt—a kinase that plays a role in the PI3K-PP2A transduction pathway that influences cellular proliferation—in 3T3 cells; this is predicted to occur through tAg-mediated alteration of PP2A activity.

A 3T3 cell line which expresses tAg only, as well as cells which express T+/t+/T′+, T+/P99At+/T′+, or T+/C157At+/T′+, were used to examine interactions
between tAg and the Rb family of proteins. These studies revealed that tAg interacts with both p130 and p107. The P99A and C157A mutations did not detectably alter the binding of tAg to either cellular protein.

Phosphorylation of specific ser/thr sites regulates multiple functions of many proteins. tAg appears as multiple forms in protein extracts of dividing 3T3 cells subjected to immunoprecipitation and Western blots; here I have determined that these bands represent tAg in both phosphorylated and unphosphorylated forms.

The experiments performed here indicate that tAg interacts with PP2A, an important regulator of cell signal transduction pathways, and unlike SV40 tAg, binds Rb proteins and contributes substantially to viral DNA replication.
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**ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>AIG</td>
<td>Anchorage-independent growth</td>
</tr>
<tr>
<td>ATM</td>
<td>ataxia telangiectasia-mutated protein</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BCIP-NBT</td>
<td>5-bromo-4-chloro-3-indolyl phosphate, nitro blue tetrazolium</td>
</tr>
<tr>
<td>BCS</td>
<td>Bovine calf serum</td>
</tr>
<tr>
<td>BKV</td>
<td>BK Virus</td>
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<tr>
<td>BME</td>
<td>Eagle’s basal medium</td>
</tr>
<tr>
<td>CAT</td>
<td>Chloramphenicol acetyl transferase</td>
</tr>
<tr>
<td>Cdk</td>
<td>Cyclin-dependant kinase</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>CPE</td>
<td>Cytopathic effect</td>
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<tr>
<td>DFA</td>
<td>Dense focus assay</td>
</tr>
<tr>
<td>DMEM</td>
<td>Delbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>EGR-1</td>
<td>Early growth response-1 protein</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>HEAT</td>
<td>Huntingtin, elongation factor 3, PP2A A subunit, and TOR kinase</td>
</tr>
<tr>
<td>HRR</td>
<td>Homologous recombinant directed DNA repair</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like growth factor 1</td>
</tr>
<tr>
<td>IP/WB</td>
<td>Immunoprecipitation/Western Blot</td>
</tr>
<tr>
<td>IRS-1</td>
<td>Insulin receptor substrate 1</td>
</tr>
<tr>
<td>JCV</td>
<td>JC Virus</td>
</tr>
<tr>
<td>KIV</td>
<td>KI Virus</td>
</tr>
<tr>
<td>LB</td>
<td>Luria broth</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCV</td>
<td>Merkel cell polyomavirus</td>
</tr>
<tr>
<td>MEF</td>
<td>Murine embryo fibroblasts</td>
</tr>
<tr>
<td>mPyV</td>
<td>Murine polyomavirus</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>ND10</td>
<td>Nuclear domain 10</td>
</tr>
<tr>
<td>NFAT4</td>
<td>Nuclear factor of activated T-cells protein</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localization signal</td>
</tr>
<tr>
<td>nt</td>
<td>Nucleotide</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymer chain reaction</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PHFG</td>
<td>Primary human fetal glial</td>
</tr>
<tr>
<td>PML</td>
<td>Progressive multifocal leukoencephalopathy</td>
</tr>
<tr>
<td>PP2A</td>
<td>Protein phosphatase 2A</td>
</tr>
<tr>
<td>p.t.</td>
<td>Post-transfection</td>
</tr>
<tr>
<td>Rb</td>
<td>Retinoblastoma</td>
</tr>
<tr>
<td>REF</td>
<td>Rat embryo fibroblasts</td>
</tr>
<tr>
<td>RPA</td>
<td>Replication protein A</td>
</tr>
<tr>
<td>SDM</td>
<td>Site-directed mutagenesis</td>
</tr>
<tr>
<td>SV40</td>
<td>Simian Virus 40</td>
</tr>
<tr>
<td>TAg</td>
<td>Large tumor antigen protein</td>
</tr>
<tr>
<td>tAg</td>
<td>Small tumor antigen protein</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TCR</td>
<td>Transcriptional control region</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA buffer</td>
</tr>
<tr>
<td>TMA</td>
<td>Transformation morphology assay</td>
</tr>
<tr>
<td>WUV</td>
<td>WU Virus</td>
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CHAPTER 1

INTRODUCTION
1.1 Background

JCV and BK Virus (BKV) belong to the Polyomaviridae family of small double-stranded DNA tumor viruses. Simian Virus 40 (SV40) is the prototype member of the subgroup of primate polyomaviruses. JCV, which is distributed among the global human population (4, 150, 174), is closely related to SV40 (69% sequence homology) (62), although these viruses display significant biological differences. For example, JCV exhibits restricted oncogenic potential in cell culture, in part due to less active and highly tissue-specific promoter/enhancer signals (101, 168). In contrast, SV40 transforms many cell types derived from a range of species, including humans (4, 55, 67). In addition, JCV’s major regulatory protein, TAg, mediates viral DNA replication less efficiently than its SV40 counterpart (18, 39, 69).

Several new members of this viral subgroup of polyomaviruses have been recently recognized. Like JCV and BKV, the newly discovered KI Virus (KIV) (118, 203), WU Virus (WUV) (65) and MC Virus (MCV) (209), are found exclusively in humans. While KIV and WUV have been found in healthy individuals and in children with respiratory infections, MCV has been found to be associated with Merkel cell tumors, which are rare skin cancers (209).

Prevalence of JCV in the human population

JCV causes asymptomatic infections in over 80% of the human population (112, 115). Stable anti-viral antibody levels and frequent occurrence of viruria in seropositive patients suggest that JCV often persists through the host’s lifetime (reviewed in Frisque and White, 1992). JCV is transmitted from parents to children who cohabitate (208). The
route of transmission is unknown, but respiratory, urine-oral, and, though less likely, fecal-oral routes have been proposed. High concentrations of virus are found in urban sewage, suggesting that contaminated water is a typical source of infection (17).

The variant of JCV that circulates extensively among the general population and establishes persistent infections is called archetype JCV (5). It has one complete copy of the promoter/enhancer region. This form of the virus is frequently detected in the urine and kidneys of seropositive individuals. Patients with progressive multifocal leukoencephalopathy (PML), the clinical disease associated with JCV, carry a rearranged form of JCV. These rearranged variants of JCV have undergone deletion and duplication events affecting the transcriptional control region (TCR) to yield more active and potentially more virulent viral strains (43, 112). Unlike the archetype for which there is essentially only one example, there are multiple rearranged forms.

The natural host of SV40 is the rhesus monkey; however, this virus was inadvertently distributed to hundreds of millions of people through contaminated poliovirus vaccinations, prepared in monkey kidney cells in the 1950s (4). The prevalence and distribution of SV40 in today’s human population are not known, though SV40 has been detected in patients too young to have been received it through contaminated vaccines (25), and there are reports that suggest SV40 is transmitted horizontally among humans (94, 183).

Genomic organization and expression of early viral proteins

Polyomavirus genomes, including JCV and SV40, are approximately 5 kilobases of circular double-stranded DNA which is associated with histones in a complex of 20-22
nucleosomes (182). Each genome contains an early coding, regulatory, and late coding region (Fig. 1). The noncoding regulatory region contains the origin of DNA replication and the promoter/enhancer signals. The early region encodes five tumor proteins which interact with cellular factors to modulate the cell cycle and mediate DNA replication; the late region codes for three structural proteins involved in viral progeny formation, and one transactivation protein.

A single JCV early precursor transcript undergoes alternative splicing to create five distinct mRNAs that are translated into the five early proteins, also called tumor antigen proteins: TAg, tAg and the three T’ proteins (Fig. 2) (178). The precursor mRNA is spliced at one of two 5' donor sites at nt (nucleotide) 4771 or 4494, as well as a shared acceptor site at nt 4426, to form mature transcripts for TAg and tAg, respectively. After the TAg exon is removed, further splicing at the shared T’ donor site, nt 4274, and one of three unique T’ acceptor sites at nt 2918, 2777, or 2704, generates mature messages for the three T’ proteins, T’135, T’136, and T’165, respectively (178). SV40’s genomic organization is nearly identical to that of JCV, and the SV40 precursor mRNA is spliced to yield transcripts encoding TAg, tAg and 17 KT protein, the latter being a homolog of JCV's T' proteins (81). The major tumor protein of both viruses is TAg, and although JCV TAg has not been studied to the same extent as SV40 TAg, a number of biological functions of each protein are known.

Late viral proteins

JCV’s late region is transcribed into two major precursor mRNAs which are then spliced to form the late mRNAs VP1, VP2, and VP3. Structural proteins VP1, VP2, and
Figure 1. The JCV genome.
The inner circle represents the circular double stranded JCV (Mad1) genome of 5130 bp. The five early proteins (arcs on the right-hand side) are encoded by five alternatively spliced mRNAs and the four late proteins are expressed from two transcripts. Early proteins are first detected 2-3 days post-infection (p.i.), and late proteins are observed 4-5 days p.i. The regulatory region (RR) consists of transcription and replication signals. The direction of transcription of mRNAs is indicated by the arrows; dotted lines represent intron sequences removed prior to protein translation.
VP3 are involved in capsid formation, viral attachment, penetration, and delivery of viral DNA into the nucleus (57, 120); these three capsid proteins accumulate in a punctuate fashion in the nucleus at regions called nuclear domain 10 (ND10), also known as promyelocytic leukemia nuclear bodies, where JCV virions are efficiently assembled (153). Agnoprotein, sometimes called LP1, though temporally grouped with the late proteins, performs regulatory tasks in contrast to the other late proteins which perform structural functions. Agnoprotein influences viral gene expression, cell cycle progression, and DNA repair (45, 46, 145), and contributes to DNA replication (82). SV40 similarly expresses the late proteins VP1, VP2, VP3 and agnoprotein, and has recently been found to express a fifth late protein called VP4, which is not incorporated into the capsid but which is required to trigger the lytic release of SV40 infectious particles (44).

The major SV40 capsid protein, VP1 (~43 kDa), in association with the minor capsid proteins VP2 and VP3, makes up the 45-50 nm icosahedral capsids (95). The SV40 VP1 pentamer forms a barrel-shaped structure around a conical hollow with its wider opening towards the interior of the virus (159); either VP2 or VP3 occupies the hollow. The 72 pentamers which make up each capsid are connected to one another by C-terminal peptides extending from a VP1 molecule in one pentamer to docking sites present in two VP1 molecules of a neighboring pentamer, stabilized by calcium ions (93). Disulfide bonds between cysteine residues further link the pentamers to each other (95). These general structural features are predicted to be conserved among all polyomaviruses.
Figure 2: Translation of alternatively spliced JCV early mRNAs.
A single early precursor mRNA is alternatively spliced to yield five mature transcripts that encode tumor or regulatory proteins. TAg and tAg transcripts have one intron removed while the 3 T' mRNAs have an additional second intron removed. The latter splicing reactions utilize a shared T' donor (5') site and one of three unique (3') acceptor sites, which are denoted by nucleotide numbers. The first 132 amino acids are shared by TAg and the three T' proteins; this common region includes a J-domain, which binds the cellular chaperone protein Hsc70, as well as an LxCxE domain which binds members of the Rb family of tumor suppresser proteins. The first 81 amino acids, including the J-domain but not the LxCxE motif, are shared by tAg as well; the unique amino acids 82-172 of tAg contain a predicted PP2A-binding domain.
1.2 Virus-cell interactions

JCV and SV40 are both capable of causing either lytic infection in permissive cells, or transformation of nonpermissive cells. Although these are the two best-studied viral pathways, there are two additional possible pathways observed for SV40, and potentially for other polyomaviruses as well. Human fibroblasts are “semi-permissive” for SV40; when exposed to SV40, only a small subset of these cells produce high levels of viral proteins and display CPE at any one time (91). SV40 can also cause a persistent infection of human mesothelial cells; no CPE is seen, and SV40 DNA remains in an episomal form (59).

Lytic infection and PML

A lytic JCV infection occurs in a relatively small number of permissive cell types including oligodendrocytes, B lymphocytes, tonsillar stromal cells, and epithelia of the urogenital tract (34). The highest titres of virus are produced in PHFG cells (115). In permissive cells, viral DNA remains episomal. Viral proteins produced early in the infection modulate expression and activity of cellular proteins, which in turn regulate cell cycle progression and cell survival. Cellular machinery is then recruited which facilitates replication of the viral genome (discussed below), and expression of late genes is activated. The structural late proteins form capsids into which viral DNA is packaged, and progeny virus escape the lysed host cell to target new susceptible cells. Cell susceptibility is determined at least in part by receptor expression. JCV requires sialic acid receptors (52, 97) and serotonin co-receptors (84) for binding to a host cell to occur; a ganglioside, GT1b, may also play a role (84). After binding to the host cell, the JCV
particle then enters the cell by clathrin-dependent endocytosis (120), is carried by an early endosome to a caveosome and is deposited in the endoplasmic reticulum (ER) (125), where uncoating occurs, before translocation to the nucleus through an intact nuclear pore (111). Astrocytes have also been found to harbor viral DNA, but in some circumstances, instead of lytic infection occurring, a nonproductive abortive infection takes place. The enlarged cells resemble those observed in malignant glioblastomas (116).

JCV was originally identified after isolation from the brain of a patient diagnosed with Progressive Multifocal Leukoencephalopathy (PML) in 1971; JCV is now known to be the causative agent of this disease (116). A JCV infection of a severely immunocompromised host may lead to the clinical disease PML (78). This demyelinating disease occurs most often in AIDS or cancer patients, or in organ transplant recipients. PML is now estimated to be the cause of death for 5-8% of AIDS patients (10, 74, 75).

Oligodendrocytes, the myelin-producing cells of the CNS, are destroyed via lytic infection by JCV. Progressive dementia and other debilitating symptoms accompany PML, as a result of the multiple demyelinated lesions in the white matter of the brain. Unfortunately, reactivation of latent JCV, and onset of PML, has recently been found to be a risk in patients with multiple sclerosis or Crohn’s disease who are treated with Tysabri (natalizumab), which is thought to depress immune surveillance of the central nervous system (50, 88, 184). PML generally leads to death within a year after the onset of symptoms.
Oncogenic transformation of nonpermissive cells

In nonpermissive cells, viral DNA is integrated into the cellular genome. Only the early viral proteins are expressed, and viral DNA replication is blocked, leading to abortive infection and, potentially, oncogenic transformation of cells (78). JCV and SV40 are both highly oncogenic in rodents in vivo, and fibroblasts from several rodent species have often been used in transformation assays in vivo (18, 71, 165). JCV causes brain tumors in hamsters (115, 181), and transgenic mice expressing the early region of JCV develop tumors of neural crest origin (87). JCV is the only human virus known to cause solid tumors in primates (99, 131); intracranial inoculation of replication-competent JCV into owl and squirrel monkeys leads to the induction of tumors in almost 100% of the animals after a 12-24 month latency period (99).

Approximately 100 million Americans were inadvertently exposed between 1955 and 1963 to SV40 present in poliovirus vaccines prepared in monkey cell cultures. Some children vaccinated with SV40-contaminated oral poliovirus vaccines shed infectious SV40 in their stool for at least five weeks after receiving the vaccine (105). After its isolation in 1960 (169), SV40 was quickly found to induce tumors in hamsters, and to transform a variety of cultured cell types derived from different host species, including primary human astrocytes (55, 67, 151). Although these data raised the question of SV40’s possible contribution to human tumorigenesis, unambiguous evidence has not been obtained to indicate a role for SV40 in human cancer (4, 103). The advent of PCR technology, an extremely sensitive assay for the detection of rare nucleic acid sequences, offered a promising tool to reexamine the role of SV40 in human tumorigenesis. However, the sensitivity of this assay has led to the possibility of false-positive results
among those laboratories which have detected SV40 DNA sequences in human tumor specimens; one body of research performed by labs around the world has concluded that SV40 DNA sequences are associated with human tumors (42, 204, 205) while a second such body failed to detect SV40 DNA sequences in human tumors (100, 122, 163). Two multi-institutional studies were performed to resolve the question by examining the presence of SV40 in malignant human mesotheliomas; however, the results of the two investigations did not agree with each other (162, 173). The most recent summary from the Immunization Safety Review Committee (established by the Institute of Medicine of the National Academies) states that there is not enough evidence to accept or reject a causal relationship between SV40-containing polio vaccines and cancer (161).

JCV and SV40 DNA have each been detected in several types of human tumors, although there is no direct evidence yet for a causative role in the formation of these cancers. JCV DNA sequences encoding TAg have been found associated with samples of human medulloblastomas, the most common malignant brain tumors in children (48, 49, 64, 86), as well as other types of brain tumors (48, 49, 126), and in human tumors of the upper and lower gastrointestinal tract (58, 134). SV40 DNA has been found in an array of human tumors including brain tumors (11, 28, 85, 89), osteosarcomas (29, 72, 90, 136), malignant mesotheliomas (4), and non-Hodgkin’s lymphoma (185, 186).

**Transformation assays**

Tumorigenesis is a multi-step process. Cells acquire many different oncogenic characteristics in the progression from a healthy cell to a cancer cell. A wide range of neoplastic characteristics may be induced by cellular DNA mutations or the activity of
viral proteins, and a variety of experimental assays have been developed to test for various hallmarks of oncogenesis.

The most stringent assay is one which examines the ability of adherent cells to proliferate and form colonies in the absence of solid support. The anchorage-independent growth assay (AIG) is performed by sparsely seeding cells in semisolid agarose. The colonies which arise from cells capable of proliferating in the soft agarose are counted, and numbers of colonies arising from different cell types or oncogenic agents can be compared.

The dense focus assay (DFA) measures the ability of adherent cells to overcome contact inhibition which prevents normal cells in a monolayer of cultured from overgrowing and forming dense mounds, or foci, of cells. In this assay, cells are seeded on tissue culture plates and maintained in medium supplemented with low levels of growth factors. When the cells become confluent, transformed cells have the ability to overgrow their neighbors and form foci which are often visible to the naked eye and which can be readily counted after staining. An additional advantage of this assay is that cells which exhibit transformed characteristics can be readily isolated and propagated for further study.

Transformed cells, unlike normal cells, often grow to high cell densities in medium supplemented with low concentrations of serum or growth factors. Assays based on this growth property can be used to distinguish normal and transformed cells simply on the basis of the rate of proliferation or the saturation density attained.
Viral tropism

JCV displays restricted host, tissue and cell-specific tropisms. The virus replicates efficiently only in human oligodendrocytes, though it is capable of infecting B-cells, hematopoietic progenitor cells, kidney tubular epithelium, and tonsillar stromal cells (107). JCV preferentially interacts with host cells displaying surface N-linked glycoproteins containing terminal α(2,6)-linked sialic acid (66). The sialic acid binding pocket of the viral capsid protein VP1 is within a deep groove; the orientation of this groove, and the side chains of the amino acids that comprise it, provide a model to explain why JCV binds to receptors with straight-chain α(2,6)-linked sialic acid but not to branched oligosaccharides or to α(2,3)-linked sialic acid (66). Serotonin receptors and a ganglioside, GT1b, have also been reported to bind to VP1 (84). Unlike SV40, which transforms a variety of cell types from different species and tissues, JCV’s transforming potential is limited to rodent fibroblasts and neural cells. Sequences within the regulatory and early coding regions are responsible for this limited transforming ability (43).

JCV’s tropism is further restricted at two levels: efficient RNA transcription is limited to cells of neuroectodermal origin (154, 168, 172), and viral DNA replication is limited to cells of human origin (115, 168, 172). JCV promoter/enhancer sequences have been identified that bind specific factors found only in permissive cells. These factors include the Dead box protein DDX1, which is found at much higher levels in cells susceptible to JCV than those which are not susceptible (166, 167), and the DNA-binding transcription factor NF-1 X (152). Ectopic expression of NF-1 X in normally nonpermissive HeLa cells leads to activation of the JCV (Mad1) (8.4-fold) and JCV (Mad4) (4.6-fold) early promoters were seen, as measured using a CAT reporter gene
system (152). Interestingly, when expression of NF-1A—a member of the same family of transcription factors as NF-1X which is found at high levels in cells nonpermissive for JCV replication—is down-regulated in non-susceptible progenitor and HeLa cells, these cells become permissive for JCV (129), suggesting that members of this family of transcription factors can regulate JCV expression positively or negatively. Nuclear factor of activated T cells (NFAT4) has also been shown to promote JCV infection of cells, and JCV early proteins up-regulated its expression (102). JCV proteins also increase the expression of another cellular protein, early growth response-1 (EGR-1), which binds to the 23-base pair GGA/C-rich sequence near the JCV origin of replication, and may be important in viral pathogenesis (138). Interaction of TAg plus two species-specific cellular replication factors on the template DNA are required for initiation of SV40 DNA replication: replication protein A (RPA) and DNA polymerase α-primase (pol α)(155, 189). In a study performed using SV40 TAg, primate and human cell line extracts were capable of supporting viral DNA replication, replication in extracts of chinese hamster ovary cells was reduced 100-fold from that seen in primate and human cells, and no replication at all was reported in mouse cell extracts (92, 155). Similarly, species-specific RPA, human pol α, and topoisomerase I are required, along with TAg, for initiation of replication of DNA containing the JCV origin of replication (32, 110).

1.3 Large T Antigen

Polyomaviruses possess small genomes and therefore have limited coding capacities. Due to these relatively compact genomes, polyomaviruses largely rely on host
factors to replicate their DNA and to propagate. They have evolved to depend on cellular replication, transcription and translation machinery in order for productive viral infection to occur, by forcing cells to progress from G0/G1 into S phase of the cell cycle. TAg, the main tumor protein of JCV, is critical for this process.

The role of TAg in viral DNA replication

The JCV and SV40 TAg proteins function through their interactions with cellular proteins as well as through enzymatic activities. An early function in DNA replication is to induce the host cell to enter S-phase, so replication machinery is available to the virus (78). The ability of TAg to deregulate the cell cycle involves its binding to and inactivation of the retinoblastoma susceptibility protein, pRb, and related proteins p107 and p130 through a sequence called an LxCxE motif (18, 53, 54, 177) (Fig. 3). This interaction is required for the release of members of the E2F family of transcription factors and subsequent activation of genes necessary for cell entry into S-phase and for DNA synthesis (78). However, E2F release also requires a second sequence called the J domain, named after its resemblance both in function and sequence to cellular DnaJ chaperones. The J domain recruits and activates the cellular chaperone Hsc70, which is a member of the DnaK family of chaperones (1). Mutation of either the LxCxE or J domains reduces or abolishes JCV and SV40 TAg-mediated DNA replication (27, 146, 177). The J domain of SV40 TAg is required for efficient viral DNA replication (27).

Once TAg has prepared the cellular environment for replication, it plays a direct role in mediating initiation and elongation steps in the viral DNA replication. The SV40 and JCV TAg bind to specific sequences at the viral origin of replication (101), and
**SV40 TAg** is known to recruit the DNA synthesis machinery by binding directly to the DNA polymerase α/primase complex and to replication protein A (RPA) to initiate replication (190). Elongation is facilitated by TAg’s helicase and ATPase functions, and its interactions with topoisomerase I (78).

TAg also binds the DNA-binding domain of TEF-1, a cellular transcription factor which acts as a repressor of late viral transcription. This interaction may facilitate the early-to-late switch in viral transcription by interfering with TEF-1 DNA binding, and thus promoting transcription of the late genes and production of structural proteins to complete the viral life cycle (9).

**The role of TAg in transformation**

As noted above, TAg plays a key role in replication in permissive cells by forcing cell cycle progression. In a non-permissive cell, this same function may result in cellular transformation. In the absence of viral DNA replication and late gene expression, the production of only the early gene products leads to the inactivation of tumor suppressor protein that regulate the cell cycle and apoptosis. Stable association of the viral and cellular genome via integration may lead to loss of a cell’s ability to control its own proliferation.

Three distinct domains of TAg are thought to be crucial for oncogenic transformation. The J domain binds Hsc70 and the LxCxE domain recruits hypophosphorylated Rb proteins; the ATPase activity of Hsc70 bound to TAg acts upon the Rb proteins recruited to the LxCxE domain, leading to a conformational rearrangement of the Rb proteins and subsequent release of E2F transcription factors (1).
The free E2F proteins then promote expression of proteins which facilitate cellular entry into S phase (Fig. 3). Normally, mitogenic signals received by cells trigger expression of cyclins which interact with cyclin dependant kinases (cdks) to promote cell cycle progression. Hypophosphorylated members of the retinoblastoma family of tumor suppressor proteins bind and inactivate members of the E2F family of transcription factors, which play roles in the control of cell cycle and growth regulatory interactions; when cyclin-cdk complexes phosphorylate Rb proteins, E2F transcription factors are released and enter the nucleus to influence gene expression. Polyomavirus tumor proteins such as TAg can override normal cell signaling pathways by binding the Rb proteins and prompting unscheduled release of the E2F transcription factors. The LxCxE domain is also required for interaction between JCV TAg and insulin receptor substrate 1 (IRS1) [20]; IRS1 binds to TAg and accompanies TAg into the nucleus, where IRS1 binds Rad51 and inhibits homologous recombination directed DNA repair (HRR). This inhibition leads to an accumulation of uncorrected DNA mutations in cells expressing TAg (130, 176). Wild type TAg-IRS1 interaction also leads to activation of PI3 kinase, which in turn up-regulates phosphorylation of Akt, a member of the MAPK pathway activated by PI3K (201).

The third critical transformation domain of TAg is a carboxy-terminal bipartite region which directly binds and inactivates p53 (119, 158). Recent studies show that beyond inactivation of p53, binding of TAg by p53 is required for recruitment of CBP/p300, which is required for acetylation of TAg (21, 121), and for binding of the insulin-like growth factor 1 (IGF-1) gene. This interaction stimulates the IGF-1 signal transduction pathway and is thought to promote malignant cell growth (16).
Figure 3: Simplified model of cell cycle dysregulation by TAg.
TAg is represented by light blue, Rb is pink, E2F is yellow, and Hsc70 orange. TAg contains multiple domains and motifs: J indicates the J-domain of TAg; L, the LxCxE motif; OBD, the origin-binding domain; Zn, the zinc domain; AAA+, the ATPase domain, V, the variable region, and HR, the host-range domain. Rb represents the family of pocket proteins, pRb, p107, or p130; E2F represents a family of transcription factors E2F 1-8. Rb repression of the E2F transcription factors normally prevents entry into S phase. TAg recruits Hsc70 through the J-domain, as well as an Rb-E2F complex via the LxCxE motif. The ATPase domain of Hsc70 affects the three dimensional conformation of Rb, which then releases E2F. The free E2F then activates genes required for entry into S phase.
Recently, a sequence in the N-terminal region of TAg was shown to bind Cul7, a cullin which is part of the E3 ligase complex involved in ubiquitin degradation of proteins (2). Cul7 binding by SV40 TAg is required for anchorage-independent growth of MEFs and growth in low serum (2). Another component of the ubiquitin degradation pathway, Fbw7, binds its substrates via a phosphorylated Cdc4 phospho-degron epitope. Although SV40 TAg has such an epitope and binds Fbw7 through it, TAg is not degraded through this interaction. It has been speculated that TAg acts as a competitive inhibitor of Fbw7 and thus reduces turnover of cellular proteins involved in cell cycle regulation such as cyclin E (191). SV40 TAg also interacts with Bub1, a mitotic spindle checkpoint protein. This interaction introduces spindle checkpoint perturbation (41) and may explain TAg’s ability to induce genetic instability and aneuploidy (33, 194). When TAg-Bub1 binding is abolished via TAg mutations, SV40 no longer effects dense focus formation in Rat-1 cells (41). These interactions between SV40 TAg and cellular regulatory factors may predict similar interactions by the closely related JCV TAg.

JCV TAg does disrupt the Wnt pathway through this binding to β-catenin. This interaction leads to β-catenin stabilization, protection from degradation, and nuclear import (63). β-catenin then up regulates expression of proteins involved in cell growth and proliferation including c-myc and cyclin D.

Both SV40 TAg and tAg have been reported to increase levels of phosphorylated Akt (201, 202, 206). TAg upregulates phosphorylated Akt levels via an intact LxCxE domain, initially suggesting that interaction between TAg and one or more members of the Rb family of proteins is required for modification of Akt. However, in Rb-negative cells with depleted p107 and p130, TAg still up regulates Akt phosphorylation. The
adaptor protein IRS1 was also found to bind TAg through the LxCxE and it is this TAg-IRS1 interaction which leads to activation of Akt in human osteosarcoma cells (201).

As well as affecting phosphorylation of cellular targets, SV40 TAg itself is phosphorylated; the best-studied post-translational modifications involve two serine/threonine clusters in the carboxy- and amino-termini of SV40 TAg. Phosphorylation of Tag influences its transforming ability, oligomerization status, nuclear import, and ability to regulate viral DNA replication (7, 60, 77, 124). Similar phosphorylation sites have been identified for JCV TAg, and mutation of Thr125 yields an unstable TAg unable to support viral DNA replication (170, 171, 180).

1.4 T' Proteins

The JCV T' proteins, T'135, T'136 and T'165, were initially predicted to be TAg degradation products but were shown by Trowbridge and Frisque in 1995 to be authentic viral proteins encoded by alternatively spliced early transcripts (178). These proteins have since been found to influence TAg-mediated viral DNA replication (123), as well as transformation, by binding and inactivating the Rb family of tumor suppressor proteins (20). SV40’s 17KT protein, a homolog to the three JCV T’ proteins, contains a J domain and LxCxE domain, a nuclear localization signal (NLS), and four unique amino acids at its carboxy-terminus (144), but its roles in SV40 replication and transforming behaviors are unclear. The presence of homologs to JCV T'-related proteins in SV40 (17KT; (165)), BKV (mini T; Prins and Frisque, unpublished data), and murine polyomavirus (mPyV) (tiny T; (135)), suggests these proteins play important roles in polyomavirus infection.
Each of the three JCV T′ proteins shares a common N-terminal 132-amino acid region with TAg. In addition, T′\textsubscript{165} shares its C-terminal 33 amino acids with TAg, while T′\textsubscript{135} and T′\textsubscript{136} have 3 and 4 unique C-terminal amino acids, respectively. These C-terminal sequences are predicted to affect the structure and phosphorylation status of the T′ proteins and lead to their unique activities possessed by each.

Differences in the T′ protein functions include differential binding to some members of the Rb family of proteins; T′\textsubscript{135} interacts with p107 more efficiently than T′\textsubscript{165}, and the interaction observed between T′\textsubscript{136} and p107 was the least robust (19). In addition, there is evidence that T′\textsubscript{136} plays a greater role in viral DNA replication than T′\textsubscript{135} and T′\textsubscript{165}, and that T′\textsubscript{135} and T′\textsubscript{136} cooperate more efficiently with Ras to transform cells than does T′\textsubscript{165} (123). In cells expressing all three T′ proteins, the overall levels of p107 and p130 are reduced, and levels of hypophosphorylated forms of these cellular proteins are increased (19).

17KT and tiny T

The role of SV40 17KT in transformation is not well understood, partly due to the difficulties encountered in attempting to generate mutants deficient in 17KT. However, 17KT has been shown to interact functionally with p130, and to reduce levels of phosphorylated p130 by more than half in quiescent fibroblasts (22), suggesting that it contains a functional J domain. 17KT mediates E2F-driven transcription and cell cycle progression, leading to rescued transformation of human cells expressing TAg with a J-domain mutation (22). An additional piece of evidence that 17KT contains a functional J-domain comes from the work of Gjoerup et al., which demonstrates that 17KT is able to
complement an RB-binding mutant TAg to rescue the growth of arrested human fibroblasts (68).

Tiny T, the mPyV protein homologous to SV40 17Kt and the JCV T’ proteins, is present at low levels in infected cells, perhaps due to its short half-life (135). Little work has been done with Tiny T; however, it has been shown to contain a functional J-domain and to stimulate the ATPase activity of Hsc70. It has been suggested that the N-terminal domain of Tiny T interacts with cellular chaperone complexes to assist the other T proteins to promote viral DNA replication and cellular transformation (135).

1.5 Small Tumor Antigen

JCV tAg has only recently become a focus of study; however, some functions of the related SV40 tAg are known. Both the N-terminal portion of SV40 tAg, which is shared with TAg and the T’ proteins, and the unique C-terminal portion of tAg influence transformation functions (13). SV40 tAg stimulates mitogenic signals in the host cell which enhance TAg-mediated transformation (1, 144, 192, 200). This cooperation between tAg and TAg is especially apparent in quiescent cells or when low levels of TAg are expressed (14, 157). SV40 tAg cannot induce cellular transformation by itself (13), but has been shown to be required in conjunction with TAg for transformation of human, but not dividing rodent cells (200). Recently, SV40 tAg was shown to be critical for maintenance of high viral copy numbers in a persistent infection of human mesothelial cells; in the absence of tAg, only low levels of the viral genome are maintained, and mesothelial cells are immortalized but not transformed (59). SV40 tAg is not necessary for, but does stimulate, viral replication in permissive cells (38).
tAg and PP2A

A key interaction occurs between the SV40 and mPyV tAgs and cellular protein phosphatase 2A (PP2A) (26, 104, 109). PP2A regulates many cell activities via dephosphorylation of various substrates, and it is evolutionarily conserved in organisms from yeast to mammals. Making up as much as 1% of total cellular proteins (156), PP2A plays a dynamic role in cellular signaling (Fig. 4).

PP2A is a modular holoenzyme consisting of three subunits. The large number of regulatory subunit B isoforms direct functional specificity when joined to the core enzyme composed of subunits A and C (104, 156). For example, pools of holoenzymes with specific B subunits are formed differentially during the course of the cell cycle, and the PP2A holoenzymes with cell-cycle-specific regulatory subunits orchestrate cell cycle progression (207). There are two isoforms each of the A and C subunits, and many isoforms of the regulatory B subunit. The most common forms of PP2A include a highly active core including a catalytic C subunit called PP2AC or PPP2C, and a scaffolding A subunit called PR65 or PPP2R1 (Fig. 4A). The rod-shaped scaffolding A subunit is composed of 15 non-identical HEAT repeats. These repeats are found in huntingtin, elongation factor 3, PP2A A subunit, and the TOR kinase proteins. The 10 N-terminal HEAT repeats are responsible for binding the regulatory B subunit, while the 5 C-terminal repeats bind the catalytic C subunit. The scaffolding A subunit serves an important structural role, but also regulates which B subunit joins the AC core; single amino acid alterations in the A subunit have been shown to disrupt the binding of specific B subunits (37).
Figure 4. Interaction between SV40 tAg and PP2A.
A. A space-filling representation of the structure of subunits A and C. The rod-shaped structural A subunit is composed of 15 non-identical HEAT repeats. HEAT repeats 3-7 are responsible for binding the regulatory subunit, while carboxy-terminal repeats 11-15 bind the catalytic subunit. The scaffolding A subunit serves an important structural role, and also regulates which B subunit joins the AC core. SV40 tAg either binds the amino-terminal HEAT repeats of the newly synthesized AC core or displaces a regulatory subunit already bound. Modified from Strack et al. (160).
B. Model of SV40 tAg bound to the A subunit of PP2A. The structural A subunit of PP2A is colored blue; the J domain and the unique domain of SV40 ST are colored green and red, respectively. The two zinc ions in the ST unique domain are yellow. Modified from Cho et al. (36).
C. Simplified model of the regulation of gene transcriptional activation by interaction of SV40 small t with PP2A. tAg binds the AC core of PP2A and modulates the activity and substrate specificity of PP2A. Multiple downstream pathways affecting cell survival and proliferation are affected. Okadaic acid is a specific inhibitor of PP2A; platelet-derived growth factor (PDGF) increases levels of activated Akt. These factors are frequently used as controls in experiments examining the effect of tAg interaction with PP2A on cellular processes.
Detection of SV40 tAg interaction with cellular proteins was first determined by co-IP experiments (142, 199). These proteins were identified, through cDNA cloning and peptide sequencing, as the A and C subunits of PP2A (117, 188). Many viral proteins, including tAg of SV40 and mPyV, complex with PP2A’s AC core (31) (Fig. 4B), and some partially displace B subunits already complexed in the holoenzyme (26). SV40 tAg binds to HEAT repeats 3-6 of PP2A’s A subunit (140, 141). The addition of SV40 tAg to the AC core alters the phosphatase activity of the holoenzyme and modulates mitogen-activated protein kinase signaling pathways (31, 157), thus potentially influencing the cell cycle (Fig. 4C.) Although tAg binds to the A subunit alone, it interacts functionally with the C subunit through its J-domain; this interaction contributes to inhibition of PP2A’s phosphatase activity (35). The SV40 tAg-PP2A interaction also alters the subcellular localization of PP2A; expression of tAg leads to loss of PP2A from microtubules (113).

tAg may affect the tumor suppressor protein p53 through its effects on PP2A. Activity of p53 is regulated by post-transcriptional phosphorylation, and thus is modulated by phosphatases such as PP2A. When SV40’s tAg forms a complex with PP2A, dephosphorylation and activation of p53 is inhibited (149); p53’s transcriptional targets include many genes that are involved in cell cycle arrest or apoptosis (24, 56, 70, 79, 80, 114, 195, 197).

**Activation of Akt and the MAPK pathway**

SV40 tAg has been shown to contribute to the transformation of cells through its interaction with PP2A. This interaction is thought to stimulate the activity of PI 3-kinase
(a lipid kinase activated by growth factors, cytokines and insulin), which in turn activates the cellular kinase Akt (202, 206). However, recent evidence suggests that SV40 tAg, when expressed under the control of the SV40 promoter, does not increase levels of activated Akt to a detectable level. Previous studies showing this activation employed constructs encoding SV40 tAg under the control of an adenovirus promoter. Under these conditions, tAg was expressed at much higher levels than when the natural SV40 promoter was used (personal communication, K. Rundell). I initially predicted JCV tAg would activate Akt based on the earlier report; however, this new information raises questions about my original prediction.

Akt regulates many signal transduction pathways, and by doing so modulates both cellular proliferation and survival. Akt is recruited to the membrane through interaction with PI3-kinase and is then activated by PDK1 phosphorylation. After activation, Akt leaves the membrane and targets both cytoplasmic and nuclear substrates for survival (47). Akt inhibits apoptosis by phosphorylating and inactivating members of apoptotic pathways such as Bad (15, 51), Forkhead transcription factors (12, 23), and caspase 9 (30). Akt activity also contributes to enhanced activity of telomerase, a necessary, but not sufficient, step in the oncogenic progression of human cells (202). Akt has two phosphorylation sites: T308, in the activation loop of the kinase, and S473, at the carboxyl terminus. Phosphorylation of T308 is likely to be critical for activation (3). PP2A dephosphorylates Akt at T308, but not at S473 (106).

Interaction between SV40 tAg and PP2A in CV-1 cells has been shown to lead to activation of MEK and ERK, components of the MAPK pathway (157). The mPyV tAg does not activate phosphorylation of Akt, but does activate the MAP pathway cascade,
which is involved in proliferation, in a PP2A-dependant manner (137). SV40 tAg’s modulation of PP2A also influences protein phosphorylation cascades involved in CREB-related transcriptional activities (193), which leads to progression of the cell cycle from G1 to S phase (76, 157).

The role of JCV TAg in viral DNA replication and cellular transformation have been the focus of much study, and since their recognition as authentic viral proteins, the T’ proteins have been established as possessing unique and overlapping functions in viral DNA replication and transformation, though more work remains to be done. However, the role of JCV tAg in replication and transformation is currently not well understood. Aims of this study have been to examine the requirement of each of the T proteins for transformation and viral DNA replication, and in particular to begin exploring tAg’s contributions to these viral activities.
CHAPTER 2
MATERIALS AND METHODS
2.1 Cell culture

Transformed Rat 2 (rat fibroblast; including subcloned cell lines Rat2/5 and Rat2/7), REF (rat embryo fibroblast) and NIH-3T3 (mouse fibroblast) cell lines and PHFG cells were cultured in DMEM with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin at 37°C in 10% CO₂. Primary REF cells were cultured in DMEM with 2.25 g/L sodium bicarbonate, supplemented with 4 mM L-glutamine, 10% FBS, 100 U/ml penicillin and 0.1 mg/ml streptomycin at 37°C in 5% CO₂. U87MG (human glioblastoma) cells were cultured in MEM supplemented with 2 mM sodium pyruvate, 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin at 37°C in 10% CO₂. C3H10T1/2 (mouse embryo fibroblast) cells were cultured in Eagle’s Basal Medium (BME) supplemented with 10% FBS, 100 U/ml penicillin and 0.1 mg/ml streptomycin at 37°C in 5% CO₂.

2.2 Plasmid DNA preparation

Cesium chloride maxiprep

Cultures of bacterial cells (500 ml) were grown in Luria Broth (LB) supplemented with 33 mM KH₂PO₄, 40mM Na₂HPO₄*7H₂O, 55mM NH₄Cl, 14.52g/L casamino acids, 30g/L glycerol, and 50 µg/ml ampicillin. Optical density (OD) determined using a spectrophotometer at 590 nm; when OD reached 0.08, 0.25g of spectinomycin, an aminocyclitol antibiotic which disrupts protein synthesis, was added to each flask. Flasks were incubated at 37°C and shaken at 200 RPM for ~12 hours. Cells were harvested by centrifuging cultures at 2800 RPM for 30 minutes. Pellets were suspended in 40 ml ice-cold TE buffer (10 mM Tris and 1 mM EDTA, pH 8) and centrifuged at 2800 RPM for 20 minutes at 4°C. Pellets were resuspended in 9 ml ice-cold 25% sucrose and tubes were incubated on ice 5 minutes. Lysozyme solution was added (0.018 µg lysozyme in 900 µl 250 mM Tris-HCl, per flask) and tubes were incubated on ice 5 minutes. Ice-cold EDTA (250 mM at pH 7.5, 3.6 ml per flask), was added and tubes were incubated on ice 5 minutes. Lysis mix (0.2% TritonX-100, 62.5 mM EDTA (ph 8), 5 mM Tris-HCl (pH 8), 14.5 ml per flask) was added and tubes were incubated on ice 10 minutes, and then cultures were centrifuged at 22,000 RPM for 90 minutes at 4°C. To the supernatant from each tube 27.5 g ultrapure cesium chloride was added; tubes were rotated until contents
were homogeneous. Ethidium bromide (27.5 mg) was added to each tube, and the tubes were centrifuged at 44000 RPM for 22 hours. Under a short-wave UV light, fluorescing DNA-containing bands were extracted via syringe and needle. The ethidium bromide was extracted from the DNA through multiple washes with CsCl-saturated isopropanol. CsCl was removed from the DNA by dialyzing samples against TE buffer. DNA was quantitated using a Nanodrop 1000 Spectrophotometer (Thermo Scientific) and visualized by agarose gel electrophoresis.

2.3 G418 selection of cell lines

To create cell lines stably expressing various combinations of viral proteins, Rat2 or NIH-3T3 cells were transfected with 5 μg of plasmid viral DNA and 0.5 μg pCR3, a vector which confers G418 [geneticin] resistance (a 5:1 molar ratio). G418-resistant cells were selected in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 400 μg/ml G418. Isolated colonies arising from single cells were transferred to new plates at 14-21 days post-transfection. Lysates of these cell lines were tested for viral protein expression via immunoprecipitation and Western blot (IP/WB).

2.4 Dense Focus Assays

Tissue culture plates of dividing Rat2/5, Rat2/7, or C3H10T1/2 cells at 40-50% confluency were fed with antibiotic-free DMEM supplemented with 10% FBS and 2 mM L-glutamine. Cells were transfected with 1 μg of viral DNA per 100 mm plate. To evaluate transfection efficiencies, I compared a modified calcium phosphate protocol to commercial protocols using Lipofectamine 2000, SuperFect (Qiagen), or GeneJuice (Novagen), a BES high-efficiency transfection protocol (6), and a Transformation Morphology Assay (TMA). Twenty-four hours after transfection, medium on the cells was changed to DMEM with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin. Twenty-four hours later, medium on the cells was changed to DMEM with 3 % FBS, 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin. Medium was replaced every 3-4 days for ~6 weeks, until foci of transformed cells appeared. These cells were isolated and propagated for further study.
Remaining cells on the plates were fixed with 3.5% formaldehyde in phosphate-buffered saline (PBS; 137 mM NaCl, 2 mM KCl, 5mM Na$_2$HPO$_4$, 2 mM K$_2$HPO$_4$) for one hour, rinsed with H$_2$O, and stained with 2.5% ammonium hydroxide to facilitate counting of the dense foci.

### 2.5 DNA transfection of cells

**Commercial Transfection Reagents**

LipofectAmine2000 (Invitrogen), GeneJuice (Novagen), Transfectol (GeneChoice) and SuperFect (Qiagen) were used according to their manufacturer’s protocol. After transfection, medium was replaced with DMEM containing 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin for 24 hours. Serum in the medium was then reduced to 3% FBS for the remainder of the experiment.

**Modified Calcium Phosphate Transfection**

Cells were seeded at a density that resulted in cultures being 70-80% confluent 24 hours later when the transfection was performed. DNA/calcium phosphate precipitate solution consisting of 0.25M CaCl$_2$, carrier DNA (8 ug/100 mm plate), plasmid DNA (1 ug/100 mm plate) and water was added to an equal volume of 2X HEPES buffered solution (1.6% NaCl, 0.074% KCl, 0.038% Na$_2$HPO$_4$·7H$_2$O, 0.2% dextrose, and 1% HEPES; pH adjusted to 7.05-7.10). After mixing well and incubating at room temperature for 20 minutes, 0.5 ml of this solution was added to each 100 mm plate; plates were incubated with transfection solution for 4-24 hours at 37°C. Cells were then rinsed with DMEM, and DMEM containing 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin was added. Medium was replaced 24 hours later with DMEM containing 3% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin.

**BES high-efficiency transfection**

Exponentially growing cells were seeded the day before transfection at 5 x 10$^5$ cells/100mm plate. On the day of the transfection, plasmid DNA was mixed with 0.25M CaCl$_2$ to make the DNA/CaCl$_2$ precipitate solution. A volume of 2X BBS (50 mM N,N-
bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES), 280 mM NaCl, 1.5 mM Na$_2$HPO$_4$, and water; pH adjusted to 6.95) equal to that of the DNA/CaCl$_2$ precipitate solution was added to the solution. After mixing well, this transfection solution was incubated 10-20 minutes at room temp. Each 100 mm plate received 1 ml of this solution, which contained 20-30 µg/ml plasmid DNA; plates were then incubated for 15-24 hours in a 37°C, 3% CO$_2$ incubator. Cells were washed twice with 5 ml PBS, and 10 ml DMEM with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin was added. Cells were incubated overnight in a 37°C, 5% CO$_2$ incubator, split 1:10 to 1:30, depending on confluency, and returned to the incubator. Medium was changed the next day and every 3-4 days thereafter with DMEM containing 3% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin, until transformed cells capable of growing in low serum appeared.

**Transformation Morphology Assay**

BHK21 cells were seeded the day before transfection at 4 x 10$^5$ per 60 mm plate in DMEM with 5% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin. Cells were 30-50% confluent when transfected 24 hours later with 1 ug of plasmid DNA per plate, using the modified calcium phosphate protocol described above. Medium was changed to DMEM supplemented with 0.5% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin 1-2 days after transfection when cells reached greater than 50% confluence. Cells transfected with JCV or SV40 DNA were split 1:2 or 1:10, respectively, 24 hours later and maintained in DMEM containing 0.5% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin. When cells became confluent, they were split again, and medium was changed every 3-4 days. Cells were examined under a microscope every day for morphological signs of transformation, including elongation, increased density, and refractile appearance of the cells.

When more than 50% of cells on a plate exhibited a transformed phenotype, viral protein expression in these cells was confirmed by indirect immunoflorescent staining to visualize nuclear TAg expression. Cells were rinsed with PBS, fixed with acetone-methanol 50:50, and incubated for 45 minutes with anti-T-protein antiserum diluted 1:10 with PBS. After rinsing 3 times with PBS, cells were incubated for 45 minutes with
secondary fluorescein-conjugated antibody, washed with PBS 3 times and once with H$_2$O, and examined under a fluorescence microscope.

2.6 DpnI Replication Assays

When PHFG cells were first processed from tissues, a mixture of astrocytes, precursor oligodendrocytes, and neurons were allowed to settle onto each tissue culture plate. Once these primary cells had adhered to the plate and appeared healthy, they were trypsinized from each 100 mm plate, frozen, and stored in liquid nitrogen. Upon thawing, PHFG cells from one vial were seeded onto eight 60 mm plates, and transfected 24 hours later with 100 ng of viral DNA, using a DEAE-dextran protocol. Briefly, two days after plating, cells were rinsed twice with DMEM. Transfection solution (1 ml) consisting of 100 ng of circularized viral DNA in 0.5 ml DMEM per plate and an equal volume of DEAE-dextran solution (50 mM Tris, pH 7.5; 0.2% DEAE-dextran, molecular weight 500,000; and DMEM) was added to each 60 mm plate of cells. Cells were incubated at 37°C for 2 hours, rocking plates gently every 20 minutes. Plates were then rinsed with DMEM once, and DMEM with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin was added to each plate. The next day, medium was changed to DMEM with 3% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin, and every 3-4 days thereafter.

At time points 0, 10, 14 and 21 days post-transfection (p.t.), low molecular weight DNA was extracted from the cells using the Hirt protocol (73). Low molecular weight DNA was digested with DpnI and EcoRI in digestion buffer (0.1M NaCl, 10 mM Tris pH=7.5, 10 mM MgCl$_2$, 10 mM beta-mercaptoethanol, 0.1 mg/ml BSA). DpnI digested the input DNA of bacterial origin, but not newly replicated DNA; EcoRI linearized input and replicated viral DNA. Digestion products were electrophoresed on a 0.8% agarose gel. DNA was transferred to a Hybond-N+ nylon membrane (Amersham) using a Rapid Downward Transfer System and an alkaline transfer protocol (Schleicher and Schuell, Amersham). The DNA bound to the membrane was subjected to 5 minutes of UV light to cross-link the DNA, which was then hybridized with linear JCV DNA radiolabeled with [α-32P] dCTP (Prime-a-Gene kit, Promega). Relative replication activities of different
viral DNAs were determined by quantitating band intensities using ImageQuant 5.2 software (Molecular Dynamics) after visualization using the Typhoon PhosphorImager.

2.7 Ras Cooperation Assays

Primary REF cells were seeded onto 100 plates (5 x 10^5 cells/plate) and co-transfected with 1 μg each of Sp72Ras, a plasmid which expresses constitutively active Ras, and a DNA construct expressing a combination of early viral proteins. A modified calcium phosphate transfection protocol was used, as described above. Two days p.t., medium containing a reduced serum concentration was added to the cells to select for transformed cells. At approximately 14 days p.t., isolated colonies were picked using a micro-pipettor and P-200 pipette tips and transferred to new plates to establish cell clones. Extracts of cells were subjected to IP/WB analysis to confirm viral protein expression. The remaining cells on the plates were fixed and stained, as described above, to count numbers of colonies.

2.8 Immunoprecipitation and Western Blot Analysis

Expression of cellular or viral proteins in cells transfected with viral constructs was detected by IP/WB analysis. Cell extracts were prepared with EBC lysis 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, 5 mM sodium fluoride, 1 mM sodium vandadate, 500 mM EDTA). Protein concentrations were determined using the BioRad protein assay (BioRad).

Lysates from cells expressing viral proteins were incubated with antibodies that recognize T proteins or cellular proteins, to co-IP complexes of viral and cellular proteins. After electrophoresis and transfer of proteins to a membrane, antibodies to the viral or cellular proteins were added to the WB to detect an interaction between the T protein and a specific cellular protein partner. To form immune complexes, protein extracts were incubated with monoclonal antibody PAb 962 that recognizes JCV T proteins, and one of the following antibodies: anti-PP2A-C (clone ID6, Upstate), anti-total- or phospho-Akt (SuperArray), or anti-total- or phospho-ERK1/2 antibodies (SuperArray). The immune complexes were collected using PANSORBIN Staph A cells
(Calbiochem) or Protein A/G Agarose beads (Pierce). Although the Protein A/G Agarose beads display lower sensitivity than the Staph A cells, they also exhibit substantially less nonspecific background binding. After incubation at 4°C overnight, immune complexes were washed three times with EBC buffer supplemented with protease and phosphatase inhibitors. Protein complexes were disrupted by the addition of loading buffer and heating at 100°C for 4 minutes, then electrophoresed on SDS polyacrylamide gels (8.75-20% acrylamide) and transferred to nitrocellulose membranes of 0.2 nm pore size overnight at 30V. In some cases the iBlot dry blotting system (Invitrogen) was used according to the manufacturer’s protocol.

Membranes were blocked for two 30-minute periods in blocking buffer (1x Tris-buffered saline [TBS], 6% powdered nonfat milk and 0.1% Tween-20) with shaking. The membranes were incubated with anti-T protein antibodies PAb 962, 2001, and 2023 (additional anti-T antibodies PAb 2000, 2003, 2024 and 2030 were used in some cases for enhanced detection), anti-PP2A, anti-ERK1/2, or anti-Akt. After washing six times with wash buffer (1x TBS and 0.1% Tween-20), membranes were incubated with either alkaline phosphatase-conjugated rabbit anti-mouse IgG, alkaline phosphatase-conjugated goat anti-rabbit IgG, or HRP-conjugated anti-rabbit secondary antibody (Reliablot, Bethyl Laboratories, Inc.). For AP-conjugated secondary antibodies, incubation with BCIP-NBT allowed direct visualization of bands. For HRP-conjugated secondary antibodies, incubation with Developing Solution from the SuperSignal West Femto kit (Pierce) or Immobilon (Millipore) allowed visualization following exposure to BioMax film (Kodak).

2.9 Phosphatase treatment of proteins

Crude cell lysates were incubated with IP antibodies and PANSORBIN Staph A cells (Calbiochem) or Protein A/G Agarose beads (Pierce), at 4°C overnight. Immune complexes were then washed 3 times with EBC buffer, and pellets were suspended in a 1x solution of λ protein phosphatase buffer (50 mM Tris-HCl, 100 mM NaCl, 2 mM dithiothreitol, 0.1 mM EGTA, 0.01 % Brij 35, pH 7.5) supplemented with 2 mM MnCl₂ and 200 U λ protein phosphatase (NEB) per 1 mg protein. After incubation at 30°C for 30
minutes, pellets were washed once with EBC buffer before suspending in sample buffer and denaturing at 100°C for 4 minutes. After centrifugation the supernatant was loaded onto an SDS polyacrylamide gel and electrophoresed. Proteins were transferred to a nitrocellulose membrane, blocked with 1X TBS supplemented with 0.1% Tween-20 and 5% nonfat milk, incubated with secondary antibody, washed, and visualized as described above.
CHAPTER 3

THE ONCOGENIC POTENTIAL OF THE EARLY PROTEINS OF JCV
TAg is considered the primary transforming protein of JCV, although the T′ proteins also exhibit oncogenic and immortalizing properties (19). Each of these four proteins interacts differently with members of the Rb family of proteins (20). Although several functions of SV40 tAg are known, JCV tAg has not been well studied, and its role in transformation is not well understood.

My hypothesis is that the five JCV T proteins exhibit both unique and overlapping functions in transformation. My first two aims were to develop an assay to measure the transformation efficiencies of different combinations of JCV early proteins, and to generate a collection of DNAs which express combinations of early proteins to use in this assay. This collection of constructs will be important beyond the current project, for our laboratory and the field.

3.1 Construction and sources of expression vectors.

I have produced an extensive collection of constructs that express several different combinations of the five JCV early proteins (listed in Table 1). I have adopted a more uniform naming scheme than has been used previously for our parental plasmids. For clarity, I have also renamed plasmids previously described; when applicable, the old name is indicated in parentheses after the first mention of each construct.

JR indicates that the plasmid contains JCV’s regulatory region, SR indicates SV40’s regulatory region, and CMV indicates the cytomegalovirus early promoter sequences. T+ or T-, t+ or t-, and T′135/136/165+ or - indicate whether or not TAg, tAg, and each T′ is expected to be expressed. T′d indicates that expression of the T′ proteins was
### Table 1. Identification of DNA constructs expressing one or more viral proteins.

Viral DNAs contain JCV (Mad1) coding sequences and either SV40 or JCV promoter/enhancer signals (indicated by SR or JR) in the pBR322 vector. WT indicates that the wild-type protein is expressed, MU that a mutant protein is expressed. H42Q indicates the protein expressed contains a His to Gln mutation at residue 42, in the J-domain; P99A, that the protein expressed contains a Pro to Ala mutation at residue 99, which is a conserved proline; and C157A, that the protein expressed contains a Cys to Ala mutation at residue 157, which is in an LxCxE domain. $T'_a$ signifies that expression of one or more of the three $T'$ proteins was abolished by point mutation of their individual acceptor splice sites; $T'_d$ means that expression of all three $T'$ proteins was abolished by a point mutation of their common donor splice site.

<table>
<thead>
<tr>
<th>Construct name:</th>
<th>TAg</th>
<th>tAg</th>
<th>T'_{135}</th>
<th>T'_{136}</th>
<th>T'_{165}</th>
</tr>
</thead>
<tbody>
<tr>
<td>SR: T+/t+/T'+</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>pSR: muT+/t+/T'+</td>
<td>MU</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>pSR: T+/t-/T'+</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>pSR: muT+/t-/T'+</td>
<td>MU</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>pSR: T'_135-, 136+, 165+</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>pSR: T'_135+, 136-, 165+</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
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<td>WT</td>
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<tr>
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</tr>
<tr>
<td>pSR: T'135-, 136+, 165+</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>pSR: T'/P99At+/T'+</td>
<td>WT</td>
<td>P99A</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>pSR: T'/C157At+/T'+</td>
<td>WT</td>
<td>C157A</td>
<td>WT</td>
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<td>WT</td>
</tr>
<tr>
<td>pSR: H42QT+/H42Qt+/H42QT'+</td>
<td>H42Q</td>
<td>H42Q</td>
<td>H42Q</td>
<td>H42Q</td>
<td>H42Q</td>
</tr>
<tr>
<td>pJR: T'/H42Qt+/T'-</td>
<td>H42Q</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
abolished by mutation of the shared T’ donor splice site; T’a indicates that expression was abolished by mutation of one or more of the individual T’ acceptor splice sites. T-/t+/T’- signifies that the tAg intron has been removed, abolishing expression of TAg and the T’ proteins since the acceptor splice site for the first intron of both TAg and the T’ proteins has been eliminated. “Mu” before T indicates that the construct encodes a truncated TAg of 410 base pairs, which is predicted to be nonfunctional. A single amino acid mutation, such as alteration of the histidine at residue 42 of tAg to glutamine, is indicated by the single letter abbreviations of the wild-type and the altered amino acids bracketing the residue number; the name of the altered protein follows. For example, SR:T+/H42Qt+/T’+ expresses wild-type TAg and T’ proteins and the mutant H42Q tAg, under the direction of SV40’s transcriptional signals.

**Parental constructs used to make recombinant DNAs prepared for this study.**

CMV:T+/t+/T’+ (previously called CMV-JCV_E (19)) expresses the entire JCV early region via the CMV early promoter in the pCR3 vector (Invitrogen). CMV:T+/t-/T’- (previously called CMV-ΔT’ (19)) expresses TAg only. CMV:T+/t-/T’+ (previously called CMV-TAg (19)) expresses TAg and the three T’ proteins, but not tAg.

The plasmid pSR:T+/t+/T’+ (previously called pSRMMT (18)) expresses all JCV wild-type early proteins under the control of the SV40 regulatory region. pJR:T+/t+/T’+ (previously called pM1TCR1A) contains the entire JCV (Mad-1) genome (132) in the pBR322 vector, and pSV40 contains intact sequence of SV40 strain 776 in the pBR322 vector.
Constructs produced for work conducted in this thesis.

pJR:T+/t-/T'_d-

TAG cDNA sequences, including the mutated T' donor splice site, were isolated from CMV:T+/t-/T'_d- and ligated into the corresponding region of pJR:T+/t+/T'+ to generate pJR:T+/t-/T'_d- (Fig. 5A-C). After correct orientation of the insert was verified by restriction enzyme analysis, the region of interest was sequenced (Nucleic Acid Facility, Penn State University) to confirm the expected construct had been produced.

pSR:T+/t-/T'_d-

pJR:T+/t-/T'_d- was digested with NcoI, which cleaves at two sites flanking the regulatory region. The corresponding SV40 fragment containing viral regulatory sequences was isolated after NcoI digestion of pSR:T+/t+/T'+ and ligated to the T+/t-/T'_d- backbone to generate pSR:T+/t-/T'_d- (Fig. 5D). The orientation of the insert was verified by restriction enzyme analysis and the expected construct confirmed by DNA sequencing.

Detailed maps describing the construction of additional plasmids expressing different combinations of viral proteins identified below are included in the appendix.

pJR:muT+/t+/T'+

pJR:T+/t+/T'+ was digested with AflII, which cleaves the DNA twice to excise a 734 base pair fragment within the unique TAg coding region, and self-ligated to produce pJR:muT+/t+/T'+. The deletion of the AflII-AflII fragment was confirmed by restriction enzyme analysis and DNA sequence analyses.
A.

Fragment sizes seen on a gel:
- 723 of pCR3/5019 of JCV
- Neol 4980
- 356 Neol
- 1284
- 273 of JCV
- 373
- 5019 of JCV
- 2783
- 2148
- 1284
- 697
- 2473 of JCV

20 bp of adapter sequence, then m 759 of pCR3

CMV:T+/t+/T'⁺
7285 bp

Mutated T' donor splice site

B.

Fragment sizes seen on a gel:
- 5053
- 2382
- 1721 of JCV
- 1629
- 425

1721 of pBR322

pJR:T+/t+/T'⁺
9490 bp

1732 of JCV

4361 of pBR322

Ligation of TAg/mutated T' donor site fragment into pJR:T+/t+/T'⁺ backbone to create pJR:T+/t- T'⁻.

NcoI 4980

PstI 3355

1/1530 of JCV

1721 of JCV

1 of pBR322

NcoI 275

3607 PstI

NcoI 4980

PstI 3350

1722 of JCV

4361 of pBR322

NcoI 4980

PstI 3355

Mutated T' donor splice site

<---1284-->
Figure 5. Generation of the recombinant DNAs pJR:T+/t-/T_d- and pSR:T+/t-/T_d'-plasmids.

A. CMV:T+/t-/T_d- and pJR:T+/t+/T'+ were both digested with NcoI and PstI. The fragments highlighted in red were gel-isolated and purified, then ligated together in a 4-fragment ligation to create pJR:T+/t-/T_d-.

B. The fragment containing the region of interest (including the removed TAg exon and the mutated T_d' donor splice site) was isolated from the parental CMV plasmid and ligated into a pJR:T+/t+/T'+ backbone lacking the corresponding fragment.

C. Since the PstI-PstI and the NcoI-NcoI fragments could be inserted in either orientation, pJR:T+/t-/T_d- clones were screened via AflIII/ScaI digestion. Possible digestion patterns are indicated. Once a correct clone was identified with this screen, the DNA was sequenced for confirmation and stored at -80°C.

D. pJR:T+/t-/T_d- was digested with NcoI, which cuts twice at sites flanking the JCV regulatory region (JR). The 8720 bp backbone fragment was gel-isolated and purified. The 427 bp SV40 regulatory region (SR) fragment (digested and gel-purified with NcoI from pSR:T+/t+/T'+) was ligated into this backbone to produce pSR:T+/t-/T_d-, the final product. After DNA sequencing was performed for confirmation, the correct DNA was stored at -80°C.
pSR:μT+/t+/T′+

pJR:μT+/t+/T′+ was digested with NcoI, which cleaves two sites flanking the JCV regulatory region. The corresponding SV40 fragment containing viral regulatory sequences was isolated after NcoI digestion of pSR:T+/t+/T′+ ligated to the μT+/t+/T′+ backbone to generate pSR:μT+/t+/T′+. The orientation of the insert was verified by restriction enzyme analysis, and the expected construct was confirmed by DNA sequencing.

pJR:T-/t+/T′-

PCR was performed on DNA template pJR:T+/t+/T′+ using primers tAg cDNA R (5′-GAG CTT ATG GAT TTA TTA GGC CTT GAT AGG TCT GCA TGG-3′), nt 4980-4942, and tAg cDNA F (5′-GCT ATC CAT AGG TTG GCA CCT TAA AGC TTT AGA TCC CTG TAG G-3′), nt 4412-4426, 4494-4517 with 4 non-JCV nt at the 5′ end. The amplicon representing the tAg cDNA with one mutation at nt 4605 (an artifact of the PCR procedure), was isolated, sequenced, and ligated to the linear TA cloning pCR2.1 vector (Invitrogen). The resulting plasmid was digested to isolate the tAg cDNA, which was ligated to CMV:T+/t+/T′- from which the corresponding wild-type sequence had been removed, to produce CMV:T-/mut+/T′-.

Site-directed mutagenesis was employed to generate a construct with the wild-type tAg sequence. CMV:T-/mut+/T′- was used as a template in a PCR reaction with complementary primers WTtAgL (5′-GCA ATC AAA GCA ATA GCA ATC TAT CCA CAC AAG TGG GC -3′), and WTtAgE (5′-GCC CAC TTG TGT GGA TAG ATT GCT ATT GCT TTG ATT GC-3′). The primers represent wild-type tAg cDNA sequence from
nt 4591-4628; the bolded wild-type nucleotides correspond to those which were altered in the original PCR amplicon, here used as a template. The PCR-generated tAg coding region was sequenced and the wild-type tAg cDNA was confirmed. The wild-type tAg cDNA sequences, including the wild type T’ donor splice site region, were removed from the vector by digestion with EcoRI and PflMI and ligated into a CMV:T+/t+/T’- backbone with the corresponding region removed, to generate CMV:T-/t+/T’-. This ensured that any secondary mutations inadvertently established via PCR would not be present in the final construct. Next, the tAg cDNA region was isolated from CMV:T-/t+/T’- and ligated to pJR:T+/t+/T’+ with the corresponding sequence removed, to create pJR:T-/t+/T’-. The final structure of pJR:T-/t+/T’- was confirmed by restriction enzyme and DNA sequence analyses.

pSR:T-/t+/T’-

pJR:T-/t+/T’- was digested with NcoI, which cleaves two sites flanking the JCV regulatory region. The corresponding SV40 fragment containing viral sequences was isolated after NcoI digestion of pSR:T+/t+/T’+ and ligated to the T-/t+/T’- backbone to generate pSR:T-/t+/T’-. The orientation of the insert was verified by restriction enzyme analysis, and the expected construct confirmed by DNA sequencing.

pSR:T-/t+/T’d-

Site-directed mutagenesis was used to alter pSR:T-/mut+/T’d- (described below) to pSR:T-/t+/T’d-. The template, pSR:T-/mut+/T’d-, was amplified and altered in a PCR reaction using complementary primers WTtAgE and WTtAgL, described above, which
span the wild-type tAg coding sequence from nt 4591-4628. The PCR product, after *DpnI* digestion and visualization on a gel, was transformed into competent *E. coli* cells. After screening ampicillin-resistant bacterial colonies, a pSR:T-/t+/T′d-clone was sequenced to confirm its identity and digested with *EcoNI* and *PflMI* to sequester the sequenced region including the wild-type tAg cDNA sequence (with the tAg intron removed), and the mutated T' donor splice site. This DNA fragment was ligated to a pSR:T-/mut+/T′d- backbone with the corresponding region removed. This ensured that any secondary mutations inadvertently established via PCR would not be present in the final construct. The orientation of the fragment was established by restriction enzyme analysis and the expected construct confirmed by DNA sequencing.

pJR:T-/t+/T′d-

pSR:T-/t+/T′d- was digested with *NcoI*, which cleaves two sites flanking the SV40 regulatory region. The JCV regulatory region was isolated after *NcoI* digestion of pJR:T+/t+/T′+ and ligated to the T-/t+/T′d- backbone to generate pJR:T-/t+/T′d-. The orientation of the insert was verified by restriction enzyme analysis, and the expected construct confirmed by DNA sequencing.

pJR:T-/mut+/T′-

The DNA fragment containing the mutant tAg cDNA was isolated from CMV:T-/mut+/T′-, which was generated as described above. This fragment was ligated to a pJR:T+/t+/T′+ backbone with the corresponding fragment removed, to generate pJR:T-
/mut+/T'- The correct insertion of the fragment was confirmed by restriction enzyme and DNA sequence analyses.

pSR:T-/mut+/T'-

pJR:T-/mut+/T'-- was digested with *NcoI*, which cleaves two sites flanking the JCV regulatory region. SV40’s regulatory region was isolated after *NcoI* digestion of pSR:T+/t+/T'+ and ligated to the T-/mut+/T'- backbone to generate pSR:T-/mut+/T'. The orientation of the insert was verified by restriction enzyme analysis, and the expected construct confirmed by DNA sequencing.

pJR:T-/mut+/T'd--

CMV:T-/mut+/T'd-- was generated as described previously. The mutant tAg cDNA region, including the mutated T' donor splice site, was isolated from the CMV vector and ligated to pJR:T+/t+/T'+ with the corresponding sequence removed, to create pJR:T-/mut+/T'd-. Proper construction of the recombinant DNA vector was confirmed by restriction enzyme and DNA sequence analyses.

pSR:T-/mut+/T'd--

pJR:T-/mut+/T'd-- was digested with *NcoI*, which cleaves two sites flanking the JCV regulatory region. The SV40 regulatory region was isolated after *NcoI* digestion of pSR: T+/t+/T'+ and was ligated to the T-/mut+/T'd-- backbone to generate pSR:T-/mut+/T'd-. The orientation of the insert was verified by restriction enzyme analysis, and the expected construct confirmed by DNA sequencing.
pSR:T+/t-/T'

CMV:T+/t-/T'+ was digested Ncol and PstI to isolate the DNA fragment containing the SV40 regulatory region and TAg cDNA. This fragment was ligated to pSR:T+/t+/T'+ which had been digested to remove the corresponding sequence. After restriction analysis to ensure correct orientation of the DNA fragment into the vector, sequencing was performed to confirm the expected structure.

pJR:T+/t-/T'

pSR:T+/t-/T' + was digested with Ncol, which cleaves two sites flanking the SV40 regulatory region. The JCV regulatory region was isolated after Ncol digestion of pJR:T+/t+/T' + and was ligated to the T+/t-/T' + backbone to generate pJR:T+/t-/T'. The orientation of the insert was verified by restriction enzyme analysis, and the expected construct confirmed by DNA sequencing.

pSR:muT+/t-/T'

pSR:T+/t-/T' + was digested with AflII, which cleaves the DNA twice to excise a 734 base pair fragment within the unique TAg coding region. The large DNA fragment was self-ligated to produce pSR:muT+/t-/T'. The structure of the mutant genome was confirmed by DNA sequencing.
pJR:muT+/t-/T'+

pSR:muT+/t-/T'+ was digested with NcoI, which cleaves two sites flanking the regulatory region. The corresponding JCV fragment containing viral regulatory sequences was isolated after NcoI digestion of pJR:T+/t+/T'+ and ligated into the muT+/t-/T'+ backbone to generate pJR:muT+/t-/T'+. The orientation of the insert was verified by restriction enzyme and sequence analyses.

pSR:T+/P99At+/T'

pSR:T+/t+/T'+ was used as template in a site-directed mutagenic PCR reaction. Primers P99AR (5′-CC CTT TAT TGC AAG GAA TGG GCT AAC TGT GCC ACT AAT CC-3′) and P99AF (5′-GG ATT AGT GGC ACA GTT AG CCA TTC CTT GCA ATA AAG GG-3′) are complementary and span the JCV early coding sequence from nt 4739-4700. The region of the PCR product encompassing the mutated codon specifying amino acid residue 99 was sequenced, isolated, and ligated into pSR:T+/t+/T'+ with the corresponding region removed via digestion, to yield pSR:T+/P99At+/T'+. The orientation of the fragment was established by restriction enzyme analysis and the expected construct confirmed by DNA sequencing.

pSR:T+/C157At+/T'

pSR:T+/t+/T'+ was used as template in a site-directed mutagenic PCR reaction. Primers C157AF (5′-CCC AAG AAG CTC TTC ATT CTC GGG AGA AAG TTC TTG G-3′) and C157AR (5′-CCA AGA ACT TTC TCC CAG GAA TGA AGA GCT TCT TGG G -3′), are complementary and span the JCV early coding sequence from nt 4562-
The region of the PCR product encompassing the codon specifying amino acid residue 157 was sequenced, isolated, and ligated to pSR:T+/t+/T′+ with the corresponding region removed via digestion, to yield pSR:T+/C157At+/T′+. The orientation of the fragment was established by restriction enzyme analysis and the expected construct confirmed by DNA sequencing.

pJR:T-/H42Qt+/T′-

pJR:T-/t+/T′- was used as template in a site-directed mutagenic PCR reaction with the primers H42QF (5′-CCCCACCTTTATCAGGTTGAGTTCTTTGC-3′) and H42QR (5′-GCAAAGAACTCCAACCTGATAAAAGGTGGGG-3′). The region of the PCR product spanning the H42Q mutation was sequenced, isolated, and ligated to a pJR:T-/t+/T′- backbone which had the corresponding fragments removed via digestion. The orientation of the fragment was established by restriction enzyme analysis and the expected construct confirmed by DNA sequencing.

pSR:H42QT+/H42Qt+/H42QT′+

pSR:T+/t+/T′+ was used as template in a site-directed mutagenic PCR reaction with the primers H42QF (5′-CCCCACCTTTATCAGGTTGAGTTCTTTGC-3′) and H42QR (5′-GCAAAGAACTCCAACCTGATAAAAGGTGGGG-3′). The region of the PCR product spanning the H42Q mutation was sequenced, removed by restriction enzyme digestion, and ligated to a pSR:T+/t+/T′+ backbone which had the corresponding fragments removed via digestion. The expected construct was confirmed by restriction enzyme and DNA sequence analyses. Residue 42 is present in the N-terminal sequences.
shared by all five JCV early proteins; the H42Q mutation thus affects all proteins expressed from this construct.

3.2 JCV early proteins cooperate with Ras to generate transformed REF cell lines.

Prolonged exposure of primary rodent cells to the constitutively active oncogene Ras accelerates the senescence process, but co-expression of a viral tumor protein such as TAg with Ras can amplify the transformation potential of the viral protein and allow the cells to bypass growth arrest and exhibit immortalized and/or transformed phenotypes (8). Primary REF cells were transfected with 1:1 ratios of the Ras-expressing Sp72-ras plasmid and plasmids expressing various combinations of JCV T proteins, to generate stable REF cell lines expressing JCV early proteins, and to compare their transforming potential.

After transfection, cells were maintained for two weeks in low serum, and those developing into clearly visible foci were selected. Cells were expanded in number, and either frozen in liquid nitrogen or lysed to examine viral protein expression. Several cell lines each were isolated that expressed various combinations of viral proteins (Fig. 6). These cells express higher levels of viral proteins than the Rat 2 cell lines I generated through G418 selection. Although the Ras cooperation assay was not expected to provide a reliable quantitative measure of the transforming potential of combinations of JCV tumor proteins, it does provide transformed cells for analysis, and allows assessment whether a viral protein provides the necessary signals to convert cells to a transformed and immortalized state.
3.3 Development of a dense focus assay to measure transformation by JCV early proteins.

The dense focus assay (DFA) depends upon transformed cells’ loss of density-dependant growth inhibition. This property leads to the ability of the cell to overgrow neighboring cells and form dense colonies or foci on a monolayer of normal cells. Our laboratory has relied upon the DFA to measure the efficiency of JCV-induced transformation and to isolate transformed cells for analysis. Initially I intended to perform the DFA to compare transformation efficiencies of various cell lines expressing viral proteins. However, in past years this assay has become less sensitive in our hands, and it has become difficult to measure JCV-induced transformation; therefore my intention became to first restore this important assay for our laboratory, and then to use it to compare transforming efficiencies of various T-protein-expressing recombinant DNA constructs. We suspect that the reduction in our assay’s utility is due, in part, to the quality of FBS now on the market. Other variables may include the susceptibility of established cell lines to transfection methods and reagents, which may change slightly with repeated passages over time. To enhance the sensitivity of the assay, I compared transformation efficiencies using different cell lines, transfection protocols and reagents. I first compared the transformation efficiencies of Rat 2/7 cells, which have been successfully used for the DFA in the past, and REF52 cells, a second rat fibroblast line which maintains a very flat morphology. I used SV40 DNA as a positive control, since it
Figure 6. Viral proteins are expressed from cells generated in a Ras cooperation assay.
DNA constructs encoding early JCV proteins were co-transfected with a mutant Ras expression vector into primary REF cells. Transformed cells were selected by maintenance in low-serum growth medium. Isolated colonies were transferred to new plates multiple times to establish clones of each cell line. Extracts of cells were then subjected to immunoprecipitation and western blot analysis to confirm viral protein expression.
A. Cell lines express all five T proteins (first lane), or TAg and tAg plus either one or two of the three T' proteins, or REF, rat embryo fibroblasts that do not express viral proteins.
B. Cell lines express all five T proteins (first lane), a tAg deletion mutant which expresses TAg and the three T' proteins only (lane 2), or a tAg deletion mutant which expresses a TAg mutant predicted to be a nonfunctional truncated protein of 410 bp, plus wild-type T' proteins (lane 3). The truncated TAg is not detectable on western blots, indicating that this mutated protein is most likely unstable.
transforms rodent cells much more effectively than JCV DNA, and compared it to JCV DNA under the control of SV40’s promoter/enhancer region; this chimeric plasmid is called pSR:T+/t+/T′+. Previous studies indicated that pSR:T+/t+/T′+ transformed Rat 2 cells approximately 30-fold better than wild-type JCV DNA. By substituting SV40 for JCV promoter-enhancer sequences, JCV early protein expression is increased, and in the past the numbers of transformed cells have been increased to a readily measurable level (~30-40 foci per plate). Calf thymus DNA, used as carrier DNA in the modified calcium phosphate assay, was used alone as a negative control. I also compared two methods of transfection: a standard modified calcium phosphate protocol, and an Invitrogen protocol utilizing the commercial reagent Lipofectamine 2000. Commercial transfection reagents such as Lipofectamine 2000, and others described below, may exert more cytotoxic effects on the cells than the modified calcium phosphate transfection protocol, but also may allow higher transfection efficiency. All experiments were carried out in triplicate. After maintaining the cells in medium containing low serum concentrations (3%) for 6 weeks to select for transformed cells which would readily grow in conditions of reduced growth factors, I stained the plates and counted foci. There were fewer dense foci on the REF52 plates than on the Rat 2/7 plates for both DNA types. The cells transfected using Lipofectamine 2000 exhibited more foci than those transfected using calcium phosphate (Table 2), but neither transfection method yielded enough foci on the positive control plate to indicate that the assay would have the required sensitivity to accurately measure differences in transformation efficiency between wild type and mutant JCV DNA constructs.
Next I compared two clones of the Rat2 cell line, Rat 2/7 and Rat 2/5. The Rat 2/5 cells have been passaged fewer times than the Rat 2/7, and thus I expected that they would exhibit a lower background to dense foci than Rat 2/7 cells, and possibly higher sensitivity to transformation. The numbers of foci seen on SV40-transfected cells were higher with Rat 2/5 cells than with Rat 2/7, though not high enough to constitute an effective assay. I performed a second experiment with these two cell lines and confirmed that neither cell type yielded numbers of foci that would permit detection of transformation activities of mutant viral early coding regions (Table 2).

The mouse fibroblast line C3H10T1/2 do not undergo detectable spontaneous transformation. These cells are very sensitive to contact inhibition (128), and are highly susceptible to transformation (133). I compared these cells to Rat 2/7 cells using the modified calcium phosphate and Lipofectamine 2000 transfection protocols. Cells were transfected with pSV40, pSR:T+/t+/T’+, or carrier DNA alone. I observed higher numbers of foci for pSR:T+/t+/T’+-transfected C3H10T1/2 cells compared to Rat 2/7 cells, but there was also a higher background of foci on the C3H10T1/2 negative control plates in this assay. I next compared C3H10T1/2 cells to R2/5 cells. I transfected both cell types with pSV40 or pSR:T+/t+/T’+, or carrier DNA alone, using the modified calcium phosphate transfection protocol. However, after 6 weeks, no plate exhibited more than six dense foci. A second mouse fibroblast cell line, NIH/3T3 cells, is frequently used in transformation studies because the cells can be transfected with high efficiency and stably maintain a normal morphology in the absence of virus (40). I compared activity in NIH3T3 vs Rat 2/5 cells; the 3T3 cells developed considerably fewer foci than Rat 2/5 cells when these two cell types were transfected with pSV40 or pSR:T+/t+/T’+ DNAs.
Table 2. Comparison of the sensitivities of various cell lines to transformation by JCV early proteins. The column labeled “Tfxn type” indicates what transfection reagents were used; Ca PO₄ indicates the modified calcium phosphate protocol described in the Materials and Methods, and commercial reagents were used in accordance with the manufacturer’s protocol. LF2000 indicates Lipofectamine 2000 was used. DNAs transfected into cells included SV40 DNA in a pBR322 vector (SV40), pSR:T+/t+/T′+(JCV), or calf thymus DNA (CT; carrier DNA) only as a negative control. R2/7 and R2/5 indicate Rat 2/7 and Rat 2/5 cell lines; C3H indicates C3H 10T1/2 cells; and 3T3 indicates NIH/3T3 cells. Each number of foci represents the average number of foci on three plates seeded and transfected in triplicate.
Reasoning that a more effective transfection protocol and/or reagent could potentially boost transfection efficiency sufficiently for my purposes, I compared the modified calcium phosphate protocol to several protocols using commercial transfection reagents: Lipofectamine 2000 (Invitrogen), SuperFect (Qiagen) and GeneJuice (Novagen) in Rat 2/5 cells. Neither the calcium phosphate nor any of the commercial protocols/reagents supported the induction of foci by viral DNA at levels that would permit reproducible quantitation of transformation efficiencies with the wild type constructs, pSR:T+/t+/T'+, or pSV40. In the absence of efficient transformation with wild type viral DNA, it was not reasonable to expect I would obtain measurable numbers of foci with the mutant viral constructs. As one last attempt to identify an appropriate transfection protocol, I again transfected pSV40, pSR:T+/t+/T'+, and carrier DNA into Rat 2/5 cells using a “high-efficiency” protocol (6), which employs a BES-containing transfection mix. However, this assay produced only a few foci per plate (Table 3).

Although 1 μg of plasmid DNA per 100 mm tissue culture plate is routinely used in transformation assays involving near-confluent cells, I tested whether increasing the amount of DNA in the transfection mix would increase transformation efficiency of Rat 2 cells. Rat 2/5 and Rat 2/7 cells were transfected with 1 μg vs. 5 μg of plasmid DNA per plate. Plates transfected with 5 μg of pSR:T+/t+/T'+ DNA produced more foci than those transfected with 1 μg. I repeated this experiment with 3, 4, or 5 μg of plasmid DNA per plate and confirmed that 5 μg led to the highest numbers of foci, although even on the plates which received 5 μg of DNA, the total number of foci was not high enough to indicate sufficient sensitivity of the assay. Furthermore, my results were not reproducible;
<table>
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<th>Assay 7</th>
<th>Assay 8</th>
<th>Assay 9</th>
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<td>T+/t+/T', 3%, 1%</td>
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<td>T+/t-/T+, 3%, 1%</td>
<td>0</td>
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<tr>
<td>CT, 3%, 1%</td>
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Table 3. Efficiencies of different assays to measure transformation by JCV early proteins. The first column under assay 7 represents numbers of foci generated by a modified calcium phosphate transfection protocol; the second column represents those generated by a “high efficiency” transfection (6). In assay 8 and 9, the amount and type of DNA transfected into 100-mm plates of cells is indicated. SV40 indicates SV40 DNA sequences in a pBR322 vector, T+/t+/T′+ indicates wild type JCV coding sequences under the control of SV40 promoter/enhancer signals, T+/t-/T′+ indicates TAg and the T′ proteins are produced under the control of SV40 promoter/enhancer signals, muT+/t+/T′+ indicates that a truncated, unstable TAg and wild type tAg and T′ proteins are produced under the control of SV40 promoter/enhancer signals, and CT indicates calf thymus DNA (carrier DNA) only, used as a negative control. In assay 10, percentages refer to amount of FBS in the maintenance medium from day 2 p.t. until 1:10 dilution (at day 21 p.t.), and then from day 21 to day 42 p.t. (when cells were fixed and stained). R2/7 and R2/5 indicate Rat 2/7 and Rat 2/5 cell lines. Each number of foci represents the average number of foci on three plates seeded and transfected in triplicate.
when I repeated this assay, the numbers of foci on plates receiving 5 μg of DNA were similar to those on plates receiving 1 μg of DNA (Table 3).

To develop an effective assay to measure transformation of cells by BK Virus (BKV), another member of our group recognized that transformed cells were present in the DFA, but were unable to overgrow neighboring normal cells to make dense foci. To determine if trypsinizing and replating transfected cells at a lower density allowed the detection of transformed cells, transformed Rat 2/5 cells were trypsinized at day 21 p.t., diluted 1:10, and replated. This allowed cells which were not strongly transformed to proliferate more quickly than untransformed cells and form foci, in contrast to the more stringent standard DFA. Cells were maintained in low serum (1%) after dilution and replating until foci appeared. I performed this protocol on Rat 2/5 cells using pSV40, pSR:T+/t+/T′+, or carrier DNA only, but again observed only low numbers of foci, similar to previous assays (Table 3).

Baby hamster kidney (BHK) cells have been used in our lab previously for transformation assays. These cells proliferate even in low serum (0.5%), so instead of using them in a DFA in which relative transformation efficiency is determined by the number of foci induced, I used them in a transformation morphology assay (TMA). Although this assay is less quantitative than the DFA, TMA is better suited to detecting transformation of cells that already exhibit a partially transformed phenotype (i.e. proliferation in low serum and reduced contact inhibition). In this assay, BHK-21 cells were transfected with pSV40, pSR:T+/t+/T′+, or carrier DNA only, and maintained in low serum (0.5%). When cells became confluent, they were trypsinized, diluted 1:2, and replated. Transformation is detected by increased growth rate and by observing changes
in the cells’ morphology, including an elongated and refractile appearance.
Transformation is confirmed by antibody staining of the cells and/or IP/WB analysis of
the cell lysates to detect viral tumor protein expression.

In a preliminary experiment, I performed the assay under conditions previously
used successfully by our laboratory. It was immediately apparent that cells maintained in
0.5% serum grew poorly, so I repeated the experiment using medium containing 1%
serum. The transfected cells remained healthy and proliferated in 1% serum over a period
of three months. The cells were trypsinized, diluted 1:2 and replated four times, but
neither the cells’ morphology nor their growth rate changed during this time. After the
fourth trypinization step, the cells failed to reach confluence again. The only cells for
which TAg expression was detected in the cultures using immunofluorescent staining
were those transfected with SV40.

Additionally, I performed a TMA, as well as a DFA, using PHFG cells. These
primary cells are typically used to test infectivity of JCV rather than transformation
activity, since they are one of the few cell types which efficiently supports the entire
productive life cycle of the virus. I reasoned that since these cells support JCV
replication, they might be transformed under some circumstances. PHFG transfected with
wild-type JCV DNA are susceptible to a lytic infection; however, if replication-defective
JCV genomes are introduced to these cells, transformation has been demonstrated. POJ
(PHFG cells transformed by Origin-deficient JCV DNA) cells were originally generated
by transfection of PHFG with JCV DNA containing a mutated replication origin. Early
viral proteins were produced under these conditions, but viral DNA replication was
blocked, leading to the transformation of the cells. In the current study I took advantage
of the observation that the JCV TAg is unable to initiate DNA replication from the SV40 replication origin when a chimeric DNA containing JCV proteins and the SV40 regulatory region (SR:T+/t+/T′+) is introduced into PHFG cells. However, the SV40 transcription signals drive high expression of the JCV tumor proteins. Therefore, I transfected cells with the chimeric plasmid pSR:T+/t+/T′+, expecting that viral DNA replication would not occur but that the relatively high levels of viral proteins would transform the cells.

I transfected PHFG cells with several constructs, including pSR:T+/t+/T′+, pSR:T+/t-/T′-, and pSR:muT+/t+/T′+; carrier DNA alone was used as a negative control. On day 16 p.t., each plate of cells was trypsinized, diluted 1:2, and replated into two plates. The cells in one set of plates were kept in medium supplemented with 10% serum and trypsinized, diluted 1:2 and replated when confluent for the TMA; cells in the other set of plates were maintained in medium supplemented with 3% serum for the DFA. The TMA cells were trypsinized, diluted 1:2 and replated 10 times during the 70 days of the assay. On day 70, 100% of the cells transfected with pSR:T+/t+/T′+ expressed TAg, as determined by antibody staining; no cells transfected with pSR:T+/t-/T′-, pSR:muT+/t+/T′+, or carrier DNA alone were positive for TAg expression. I observed no changes in cell morphology at any time.

At day 60 p.t., I isolated several foci from the pSR:T+/t+/T′+, pSR:T+/t-/T′-, and pSR:muT+/t+/T′+ plates in the DFA experiment, and expanded the cell numbers for IP/WB analysis. Lysates prepared from cells transfected with pSR:T+/t+/T′+ contained all five JCV early proteins. I then stained the original plates and counted the remaining
foci. There were fewer than 10 foci per plate on the plates of cells transfected with pSR:T+/t+/T′+, and no foci on any of the other plates (Table 3).

I concluded that in my hands, the DFA, or variations of it, was not sensitive enough to reliably measure transformation by JCV early proteins. I therefore terminated the experiment at this point and decided to use the collection of DNA constructs I had generated in an alternative assay.
CHAPTER 4

JCV TAG CONTRIBUTES TO VIRAL DNA REPLICATION AND INTERACTS WITH CELLULAR PROTEINS
4.1 tAg’s role in promoting viral DNA replication

TAg is the primary replication protein, but the role of the other four JCV early proteins is not clear. Mutants that fail to express all three T’ proteins exhibit approximately 10-fold reduction in viral replication efficiency. There is some redundancy in this system; as long as two T’ proteins are expressed, DNA replication of T’ mutants is indistinguishable from wild type. The collection of viral DNA constructs discussed in Chapter 3 allowed me to examine the contribution of each protein using the DpnI replication assay (127). I initially tested several parameters including cell type and transfection method to optimize the sensitivity of the assay.

JCV replicates most efficiently in PHFG cells, but these cells are difficult to obtain and propagate, and considerable variability in the quality of PHFG cultures is apparent. U87MG cells are a cell line derived from a human glioma-astrocyte progenitor, and I speculated that these neural cells might support viral replication nearly as well as PHFG cells. Both cell types were transfected with JCV DNA, and PHFG cells clearly supported viral DNA replication more efficiently than did U87MGs (Fig. 7A), so I employed primary cells for subsequent experiments.

I next compared replication activities using different transfection methods, and preparations of viral DNA. Cytotoxic effects may vary depending on the transfection reagent used and the cells employed. Furthermore, because DNA purity may vary with the preparation method, I also examined the effect of this parameter on transfection efficiency. I transfected PHFG cells with DNA purified via cesium chloride density centrifugation or the PureLink HiPure Plasmid Maxiprep kit (Invitrogen), and used either a DEAE-dextran transfection or Lipofectamine 2000 (Invitrogen) protocol. The DNA
prepared in cesium chloride gradients produced slightly better results than the DNA from the Invitrogen kit, and DEAE-dextran transfection was more effective than the LipofectAmine 2000 protocol (Fig. 7B).

Based on the results of the preliminary experiments, I decided to conduct my \textit{DpnI} replication assay in PHFG cells using the DEAE-dextran transfection protocol and viral DNAs prepared from cesium chloride gradients. Cells were transfected with JR:T+/t+/T'+, as a positive control; JR:T+/t-/T'-, to investigate whether TAg alone could drive replication of viral DNA; JR:muT+/t+/T'+, to verify that expression of defective TAg, even in the presence of tAg and the T' proteins, would abolish replication; JR:T-/t+/T'+, to verify that tAg alone is not sufficient to allow viral DNA replication; JR:T-/t-/T'+, to determine if the absence of tAg altered DNA replication potential; or JR:T+/t-/T'+, to verify that the T' proteins alone do not mediate DNA replication in the absence of TAg. Viral DNA replication was detected for JR:T+/t+/T'+, but not for any other construct (Fig. 8).

The absence of JCV tAg resulted in significant reduction in DNA replication potential. To follow up on this observation, I generated constructs JR:T+/P99At+/T'+ and JR:T+/C157At+/T'+, and tested these two tAg mutants using the transfection replication assay protocol employed in the first experiment. JR:T+/t+/T'+ served as a positive control, and JR:T+/t-/T'-, JR:muT+/t+/T'+, JR:T-/t+/T', JR:T+/t-/T'-, JR:muT+/t-/T'+, and JR:T+/t+/T'- were included in the experiment in addition to JR:T+/P99At+/T'+ and JR:T+/C157At+/T'+. The two constructs containing mutant tAgs, JR:T+/P99At+/T'+ and JR:T+/C157At+/T'+, did replicate; at days 7 and 10 p.t., both of these DNAs replicated
Figure 7. PHGF cells support replication of JCV DNA more efficiently than U87MG cells, and calcium phosphate transfection of viral DNA prepared with a maxiprep kit drives efficient replication of viral DNA.

A. To optimize our replication assay protocol, we tested the ability of PHFG (P) and U87MG (U) cells to support JCV DNA replication. These cells were transfected with viral DNA, and at days 0, 7, 10, and 14 post-transfection, viral DNA was extracted, digested with DpnI and EcoRI restriction enzymes, electrophoresed on an agarose gel, transferred to a nitrocellulose membrane, hybridized to P$^{32}$-labeled dCTP, visualized on a Typhoon phosphorimager, and quantitated with ImageQuant software. The marker shown in the left-hand lane is 1 nanogram of linear JCV DNA (5130 bp). Each sample was examined in duplicate.

B. To further optimize our replication assay protocol, PHFG cells were transfected with JCV or JR:T+/t+/T'- DNA prepared either via a cesium chloride maxiprep protocol, or a PureLink HiPure Plasmid Filter Maxiprep Kit (Invitrogen). Cesium chloride-purified DNA was also transfected into cells either by the DEAE-dextran or Lipofectamine 2000 (Invitrogen; indicated by (LF)) transfection protocol. At days 0, 10, 14 and 21 post-transfection, viral DNA was extracted, treated with DpnI and EcoRI restriction enzymes, electrophoresed on an agarose gel, transferred to a nitrocellulose membrane, and hybridized to [P$^{32}$]dCTP-labeled probe. Bands were visualized with a Typhoon phosphorimager and quantitated with ImageQuant software. The marker shown in the left-hand lane is 1 nanogram of linear JCV DNA (5130 bp). Each sample was examined in duplicate.
Figure 8: Replication efficiencies of JCV DNA constructs expressing combinations of early viral proteins. PHFG cells in 60 mm plates were transfected with 400 ng of JCV DNA. At each time point, viral DNA was extracted with the Hirt protocol (73). This DNA was digested with \textit{DpnI} and \textit{EcoRI} to cleave input DNA into small fragments. DNA was electrophoresed on a 0.8% agarose gel and the separated DNA fragments were transferred to nitrocellulose membranes and hybridized to \[^{32}P\]dCTP-labeled probe. Bands were visualized with a Typhoon phosphorimager and quantitated with ImageQuant software. The marker shown in the left-hand lane is 1 nanogram of linear JCV DNA (5130 bp). Each sample was examined in duplicate, using DNA extracted from cells cultured in independent plates.
better than the T’ deletion construct, JR:T+/t+/T’-, but not as efficiently as the wild type control.

I performed this assay again and compared the same group of DNAs. I observed replication of JR:T+/t+/T’+ at all time points after day 0. No replication of JR:T+/t-/T’-, JR:mT+/t+/T’+, JR:T-/t+/T’-, JR:T+/t-/T’+, or JR:mT+/t-/T’+ was observed at any time point. JR:T+/t+/T’-, JR:T+/P99At+/T’+ and JR:T+/C157At+/T’+ replicated approximately 17-, 25- and 3-fold less efficiently than JR:T+/t+/T’+, respectively (Fig. 9).

The H24Q mutant tAg construct is different from the P99A and C157A mutant tAg constructs in that the H42 residue is present in all five JCV early proteins, whereas the P99A and C157A mutations affect tAg only. An alteration in the H42 residue in tAg will also affect the TAg and T’ proteins, if they are expressed. Therefore I generated a H42Q construct which expresses only the H42Q mutant tAg, with no other early proteins. In an attempt to compare the replication potential of the H42Q mutant tAg, I co-transfected PHFG with JR:T+/t-/T’+, (encoding wild type TAg and T’ proteins) plus either JR:T-/t+/T’- (encoding wild-type tAg) or JR:T-/H42Qt+/T’- (encoding H42Q mutant tAg); however, no replication occurred in either set of cells (Fig. 10).

Lower levels of viral proteins are produced during a JCV infection than during an SV40 infection. I hypothesized that if JCV protein expression could be increased, JCV DNA replication would be elevated as well. Although the SV40 promoter-enhancer
Figure 9. The P99 and C157 residues of tAg contribute to tAg-mediated JCV DNA replication. PHFG cells in 60 mm plates were transfected with 400 ng of JCV DNA. At each time point, viral DNA was extracted with the Hirt protocol (73). This DNA was digested with DpnI and EcoRI to cleave input DNA into small fragments. DNA was electrophoresed on a 0.8% agarose gel and the separated DNA fragments were transferred to nitrocellulose membranes and hybridized to [P\textsuperscript{32}]dCTP-labeled probe. Bands were visualized with a Typhoon phosphorimager and quantitated with ImageQuant software. The marker shown in the left-hand lane is 1 nanogram of linear JCV DNA (5130 bp). Each sample was done in duplicate.
Figure 10. Co-transfection of a DNA expressing tAg with a second DNA expressing TAg and the T' proteins fails to drive JCV DNA replication. PHFG cells in 60 mm plates were transfected with 400 ng of JCV DNA. At each time point, viral DNA was extracted with the Hirt protocol (73). This DNA was digested with DpnI and EcoRI to cleave input DNA into small fragments. DNA was electrophoresed on a 0.8% agarose gel and the separated DNA fragments were transferred to nitrocellulose membranes and hybridized to [P$^{32}$]dCTP-labeled probe. Bands were visualized with a Typhoon phosphorimager and quantitated with ImageQuant software. The marker shown in the left-hand lane is 1 nanogram of linear JCV DNA (5130 bp). Each sample was done in duplicate.
signals are more potent than those of JCV, I could not use the pSR:T+/t+/T'+ construct alone to enhance JCV early protein expression and replication because JCV TAg does not productively interact with the SV40 origin of replication. Thus no viral DNA replication would take place in cells transfected with this chimeric construct even though early protein expression was enhanced. To overcome this limitation, I co-transfected PHFG cells with one of several constructs containing various JCV early protein coding regions under the control of a CMV regulatory region, plus a construct which contained the JCV origin of replication. There are two important differences between this and the previous assay; the first is promoter type—the CMV is even more potent than that of SV40—and the second is the identity of the JCV sequences present in the construct. JR:T+/t-/T'+ includes the full-length JCV genome and no vector DNA, whereas CMV:T+/t-/T'+ contains only the early region of JCV, plus a plasmid vector backbone including the CMV promoter. I transfected PHFG cells with CMV:T+/t+/T'+; CMV:T+/t-/T'+; or CMV:T+/t-/T'-, plus pM1O, which contains the JCV origin of DNA replication but no viral protein coding information. I isolated replicated viral DNA at days 0, 3, 7, and 10 post-transfection. Replicated CMV:T+/t+/T'+ was detected at all time points, while replicated CMV:T+/t-/T'- DNA was not observed at any time point. Limited replication of the small t deletion construct, CMV:T+/t-/T'+, was observed at day 3 p.t., in contrast to the first assay, in which physiologically normal (low) levels of viral proteins were expressed, and in which I did not see any viral replication of JR:T+/t-/T'+.

Since I saw the highest levels of replicated viral DNA at 3 days p.t., I repeated the co-transfection replication protocol, and extracted viral DNA at days 0, 2, 3, and 4 p.t.
The highest level of viral DNA replication was observed at day 4, but it was only slightly higher than at day 3 p.t.. Again, the same replication pattern was observed: CMV:T+/t+/T'+ DNA replicated more efficiently than did CMV:T+/t-/T'+; no replication of CMV:T+/t-/T'- was detected. Comparable levels of DpnI-digested input DNA were observed for the three DNA types. I repeated this experiment once more, extracting viral DNA at days 0, 3, 4, and 5 p.t., and obtained similar results (Fig. 11). Bands representing replicated viral DNA were visualized with a Typhoon phosphorimager and quantitated with ImageQuant software (Table 4).

4.2 Interaction between JCV tAg and PP2A.

Protein phosphatase 2A (PP2A) regulates several important cell signal transduction pathways and is critical to cellular proliferation. For small DNA tumor viruses, including the polyomaviruses, viral induction of cellular proliferation is a key step in either cellular transformation or viral DNA replication; small t-antigen proteins of several polyomaviruses interact with PP2A to modify its impact on cellular substrates. Moreno et al. (108) found that expression of SV40 tAg up-regulates 452 genes at least 1.5-fold in HEK cells, and predicted that most activation resulted from tAg’s interaction with PP2A. These genes fall into several broad categories including those which affect cellular proliferation, development and morphogenesis, inflammation, transcriptional activation, and antigen presentation (108). SV40 tAg is known to interact with the AC core of PP2A; tAg is bound by the A subunit, and functionally replaces the regulatory B
Figure 11. Transfection of JCV DNA encoding early genes into PHFG cells drives replication of a second DNA containing a viral origin of replication.

PHFG cells in 60 mm plates were transfected with 400 ng of one of the indicated DNA constructs encoding viral early genes under the control of the CMV promoter. The DNA constructs express either all five tumor proteins (T+/t+/T'+), TAg and the T' proteins only (T+/t-/T'+), or TAg only (T+/t-/T'-). A second plasmid called M10 containing JCV’s origin of replication was also transfected into the PHFG cells. The ability of proteins produced by the first plasmid to drive replication of the second plasmid in a trans fashion was tested. At each time point, viral DNA was extracted and digested with DpnI and EcoRI to cleave input DNA into small fragments. DNA was electrophoresed on a 0.8% agarose gel and the separated DNA fragments were transferred to nitrocellulose membrane and hybridized to [P³²]dCTP-labeled probe. Bands were visualized with a Typhoon phosphorimager and quantitated with ImageQuant software. The marker shown in the left-hand lane is 1 nanogram of linear JCV DNA (5130 bp). Each sample was done in duplicate.
### Table 4. *DpnI* assay to measure viral DNA replication activity.

After visualization on a Typhoon phosphorimager, Southern blot bands were quantitated using ImageQuant software; each band representing replicated viral DNA was compared to replicated JR: T+/t+/T', or replication of pM1o mediated by CMV: T+/t+/T'.

#### A. Ratios of replicated DNAs encoding subsets of early proteins under the control of the JCV regulatory region to replicated wild-type JCV genome.

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<th>D7</th>
<th>D10</th>
<th>D14</th>
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</tr>
<tr>
<td>T-/t+/T'+</td>
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<td>0</td>
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<tr>
<td>T-/t+/T'</td>
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<td>0</td>
</tr>
<tr>
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<td>0</td>
</tr>
<tr>
<td>T+/t+/T'+</td>
<td>0.03</td>
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<td>0.04</td>
</tr>
<tr>
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<td>T+/t+/T'-</td>
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#### B. Ratios of DNA replication mediated by expression of T+/t-/T' and T+/t- /T' - to DNA replication mediated by JR:T+/t+/T'.

<table>
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<th>DNA</th>
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<th>D3</th>
<th>D4</th>
</tr>
</thead>
<tbody>
<tr>
<td>T+/t+/T'+</td>
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<td>1</td>
<td>1</td>
</tr>
<tr>
<td>T+/t-/T'+</td>
<td>0.00</td>
<td>0.11</td>
<td>0.13</td>
</tr>
<tr>
<td>T+/t-/T'-</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>DNA</th>
<th>D3</th>
<th>D4</th>
<th>D5</th>
</tr>
</thead>
<tbody>
<tr>
<td>T+/t+/T'+</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>T+/t-/T'+</td>
<td>0.05</td>
<td>0.10</td>
<td>0.14</td>
</tr>
<tr>
<td>T+/t-/T'-</td>
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<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
subunit of PP2A. Through this interaction, tAg alters the ability of PP2A to modulate cellular substrates such as PI3-kinase, which is thought to stimulate activation of the Akt pathway, to enhance cell survival and proliferation. Interaction of mPyV tAg with PP2A activates the MAP kinase cascade, which also leads to cell proliferation.

A recent study showed that interaction between a JCV tAg-GST fusion protein and PP2A takes place in vitro (148). To determine whether interaction between tAg and PP2A takes place in vivo, lysates prepared from REF and 3T3 cells expressing JCV early proteins were subjected to IP/WB. The lysates of the REF and 3T3 cell lines expressing all five of the JCV T proteins and the 3T3 cell line expressing tAg alone were first IPed with anti-T antibody, to visualize the viral proteins expressed from each cell line, and then with anti-PP2A antibody, before being blotted with anti-T antibodies. The A and C subunits of PP2A are normally bound tightly to each other to form the AC core. When anti-PP2A-C antibody was used in the immunoprecipitation step and blotting was performed using anti-T-protein antibodies, tAg was detected, indicating a physical interaction between PP2A C and JCV tAg, likely occurring through the A subunit of PP2A (Fig. 12). When proteins in the lysate were immunoprecipitated with anti-T-protein antibody PAb 962 and blotted with anti-PP2A-C antibody, PP2A C is observed, indicating the interaction is specific. The interaction is stronger in lysates of REF cells compared to 3T3 cells, although the amount of tAg expressed in the REF and 3T3 cells stably transfected with SR:T+/t+/T'+ is comparable. This may be a result of lower levels of PP2A expressed in 3T3 cells compared to REF cells. Less interaction is seen between tAg and PP2A in 3T3 cells expressing tAg only, though this may be partly due to the lower levels of tAg expressed in these cells.
Figure 12. Wild-type tAg interacts with PP2A in cells expressing JCV early proteins.

A. tAg interacts with PP2A. Lysates of REF cells stably transfected with SR:T+/t+/T+ (indicated by REF) and 3T3 cells stably transfected with SR:T+/t+/T+ or SR:T-/t+/T- were subjected to immunoprecipitation with either anti-T protein antibodies or anti-PP2A antibodies. Samples were electrophoresed on 18% SDS polyacrylamide gels. Western blotting was performed using anti-PP2A antibodies.

B. Lysates of REF cells stably transfected with SR:T+/t+/T+ (indicated by REF) and 3T3 cells stably transfected with SR:T+/t+/T+ or SR:T-/t+/T- were subjected to immunoprecipitation with either anti-T protein antibodies or anti-PP2A antibodies. Samples were electrophoresed on 18% SDS polyacrylamide gels. Western blotting was performed using anti-T protein antibodies.
4.3 Interactions between tAg mutants and PP2A.

Several conserved regions of SV40 tAg are critical for binding PP2A. Two cysteine-rich CxCxxC sequences located in the unique carboxy terminal region of SV40 tAg promote conformational stability of the protein, bind zinc ions (179), and are required for binding to PP2A (35, 104). Three conserved residues—cysteine 97, proline 101, and cysteine 103—located between these two cysteine clusters are also important for interaction with PP2A (Fig. 13). When any of these residues is altered, binding to PP2A is diminished (109). tAg mutants with alterations of cysteine 97 and proline 101 were defective for binding of tAg to PP2A; point mutation of cysteine 103 reduced, but did not completely abolish, binding (109).

I generated two tAg point mutants to examine the contribution of two residues to PP2A binding. In the first tAg mutant, proline 99 (homologous to SV40 tAg’s proline at residue 101) was altered to alanine, and in the second mutant, cysteine 157 (the central residue in tAg’s newly recognized second LxCxE motif) was altered to alanine (Fig. 14). Several stably transformed cell lines expressing these mutant tAgs were tested via IP/WB for viral protein expression. Lysates of two independently generated cell lines expressing each wild-type or mutant set of JCV early proteins were subjected to co-IP/WB to examine the interactions between P99A and C157A tAgs and PP2A. Relative to wild type tAg, I did not see a decrease in binding between either mutant tAg and PP2A (Fig. 15). I also observed binding between TAg and PP2A. Expression of SV40 TAg has been reported to lead to dephosphorylation of p130, and the fact that okadaic acid, a specific inhibitor of PP2A, completely abolishes this dephosphorylation although a PP1 inhibitor
Figure 13: Comparison of JCV and SV40 tAg amino acid sequences.
The JCV tAg sequences are indicated with a blue font and the SV40 tAg is shown using a black font. The highlighted sequence comprises the unique region of each tAg protein. The two conserved cysteine clusters involved in SV40 tAg-PP2A binding are marked with boxes around them, as are two recently recognized LxCxE motifs in JCV tAg. Histidine 42, part of a conserved tripeptide region required for J domain function, proline 99, a conserved residue required for binding of SV40 tAg to PP2A, and cysteine 157, the central residue of the newly recognized second LxCxE motif in JCV tAg, are in red font and indicated by arrows. These three residues have each been mutated to generate the three tAg point mutants H42Q, P99A, and C157A.
Figure 14. Expression of early JCV proteins from 3T3 cell lines stably transfected with recombinant DNAs. 3T3 cells were stably transfected with DNA constructs expressing JCV proteins under the control of SV40 promoter/enhancer signals. Two independently generated cell lines that express each DNA construct were tested, with the exception of T-/-t+/T′--; only one cell line expressing tAg alone was tested. Lysates of these cells were subjected to immunoprecipitation with the anti-T monoclonal antibody PAb 962. Samples were then electrophoresed on 18% protein gels and transferred to nitrocellulose membranes. Western blotting was performed using a cocktail of anti-T monoclonal antibodies, including PAb 962, 2000, 2001, 2003, 2024, and 2030.
Figure 15. Interaction of the P99A and C157A mutant tAgs with PP2A.
Two independently generated cell lines expressing each subset of viral proteins were tested, with the exception of the cell line expressing tAg alone; only one of this cell line was generated.

A. Interaction between the C157A tAg mutants and PP2A. Lysates of 3T3 cell lines expressing JCV early proteins with either wild-type or mutant tAg were subjected to immunoprecipitation with either anti-PP2A or anti-T-protein antibodies. Each lysate (6 mg) was electrophoresed on an 18% SDS polyacrylamide gel. Lysates in lanes 1-5 and 7 were IPed using anti-PP2A antibody; lysate in lane 6 was IPed using anti-T-protein antibody. Blotting was performed using anti-T-protein antibodies.

B. Interaction between the P99A tAg mutants and PP2A. Lysates of 3T3 cells expressing JCV early proteins with either wild-type or mutant tAg were subjected to immunoprecipitation with either anti-PP2A or anti-T-protein antibodies. Each lysate (6 mg) was electrophoresed on an 18% SDS polyacrylamide gel. Lysates in lanes 1-4 and 6 were IPed using anti-PP2A antibody; lysate in lane 5 was IPed using anti-T-protein antibody. Blotting was performed using anti-T-protein antibodies.
does not, suggests that TAg recruits PP2A for this purpose (96), which aligns with my observation.

4.4 Interactions between tAg and members of the Rb family

The LxCxE motif which is present in JCV TAg and the T’ proteins binds members of the Rb family of tumor suppressor proteins. tAg has not previously been reported to bind to members of the Rb family. However, while performing a co-IP/WB to detect JCV early proteins interacting with p107 and p103, Bollag et al. (19) observed, in addition to bands representing TAg and the T’ proteins, a 20 kDa band which was also bound to p107 and p130. Although tAg was a likely candidate, the authors could not explain such binding because tAg was thought to lack the LxCxE motif (19). Although the LxCxE site shared by TAg and the T’ proteins occurs in sequences not shared with JCV tAg, upon reexamination of the tAg sequence, M. Reviriego-Mendoza in our lab recently recognized two LxCxE motifs in the unique C-terminal region (Fig. 13). The first of these, residues 93-97, is common to JCV and BKV but not SV40; the second motif, residues 155-159, is unique to JCV. This reopened the question regarding JCV tAg’s ability to interact with members of the Rb family.

To determine whether tAg interacts with the Rb family of proteins, we performed co-IP/WBs upon lysates of cells either expressing all five JCV early proteins or tAg alone. Lysates of cell lines expressing different mutant tAgs was also included to study the contributions of several tAg regions to the binding of p107 and p130. The C157A tAg construct contains a point mutation which alters the central cysteine of tAg’s second
LxCxE motif to alanine. This mutation in the single LxCxE domain of JCV and SV40 TAg disrupts the Rb-binding function. In the P99A tAg construct, the proline at residue 99 is altered to alanine; this conserved proline is immediately adjacent to the first LxCxE motif. G418-selected cell lines expressing these mutant tAgs, as well as wild-type tAg, were used to compare binding efficiencies to members of the Rb family.

Cell lysates were immunoprecipitated with anti-p130 or anti-p107 antibodies. Complexes were electrophoresed in an 18% polyacrylamide gel and viral proteins were detected by immunoblotting using a cocktail of antibodies that recognize the JCV early proteins. tAg was found to interact with both p130 and p107 (lanes 4 and 6, Fig. 16 and 17, respectively). tAg carrying the mutation C157A, which disrupts the second potential LxCxE domain, also interacted with both p130 and p107, suggesting that the second LxCxE motif is not required for binding to the RB proteins. The interactions between P99A tAg and p130 or p107 were less robust than those between C157A tAg and p130 or p107. This difference might be due in part to the lower expression in the T+/tP99A+/T’+ cell line relative to that in the T+/tC157A+/T’+ line.

4.5 Phosphorylation status of tAg

The phosphorylation status of serine and threonine residues clustered in the C- and N-terminal regions of JCV and SV40 TAg s influences the initiation of DNA replication (60, 171, 187) and the efficiency of transformation (180). Phosphorylation of JCV TAg’s T125 residue is critical to protein stability and viral DNA replication activity (180), while hyperphosphorylation of SV40 TAg has been shown to inactivate the DNA
Figure 16: Wild type and mutant tAgs bind p130.
JCV tAg interacts with p130. Protein extracts prepared from stably transfected 3T3 cell lines expressing all 5 JCV early proteins (SR:T+/t+/T'+), tAg only (SR:T-/t+/T'-), TAg, the 3 T' proteins and one of the two tAg mutants (SR:T+/P99A+t/T'+, SR:T+/C157At+/T'+), or 3T3 cells alone (as negative controls) were immunoprecipitated with PAb962 or anti-p130 antibodies as indicated. The proteins were separated on an 18% polyacrylamide gel and Western blot analysis was performed using a cocktail of monoclonal antibodies that recognize the JCV early tumor proteins (PAb 416, 901, 962, 2000, 2001, 2003, 2023, 2024, and 2030). IP/WB was performed by M. Reviriego-Mendoza.
**Figure 17. Wild type and mutant tAgs bind p107.**

JCV tAg interacts with p107. Protein extracts from the same 3T3 cell lines listed in the legend to Fig. 18 were immunoprecipitated with PAb962 or anti-p107 antibodies as indicated, and the immunoprecipitated proteins were separated on an 18% polyacrylamide gel. The tumor proteins were detected by Western blot analysis using a cocktail of monoclonal antibodies that recognize the JCV early tumor proteins (PAb 416, 901, 962, 2000, 2001, 2003, 2023, 2024, and 2030). IP/WB was performed by M. Reviriego-Mendoza.

<table>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T+/C157 A+/T'</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T-/C157 A+/T'</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>T+/p99 A+/T'</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
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</tbody>
</table>

**IP Ab α-T:**

- 3T3: +
- T+/C157 A+/T': -
- T-/C157 A+/T': -
- T+/p99 A+/T': +

**IP Ab α-p107:**

- 3T3: -
- T+/C157 A+/T': +
- T-/C157 A+/T': +
- T+/p99 A+/T': -
unwinding function of TAg (61). The level of phosphorylation may control the percentage of TAg proteins in a host cell that form the hexamers and are recruited to the origin of replication, vs. the percentage of TAg monomers free to interact with other cellular factors (60). The T' protein functions are also regulated by their phosphorylation status (19, 180). Phosphorylation of neither SV40 nor JCV tAg has been previously reported.

In WBs performed on lysates prepared from dividing 3T3 cells stably transfected with SR:T+/t+/T'+, I observed two or more bands representing tAg. Analysis of tAg sequence by PredPhospho software (83) predicts four potential phosphorylation sites for the tAg amino acid sequence, at serine 10, threonine 92, threonine 165, and tyrosine 167, but it is currently unknown whether any or all of these sites are phosphorylated at any time in the viral life cycle, or what effect modification of these sites might have on tAg function. Upon treatment of lysates of these cells with λ protein phosphatase, only the fastest migrating band remains (Fig. 18). Multiple tAg bands remain in the untreated lysates, indicating these represent phosphorylated and un- (or under-) phosphorylated forms of tAg. Similarly, treatment with λ protein phosphatase reduces multiple bands representing phosphorylated forms of T'_{135}, T'_{136} and T'_{165} to the single fastest migrating band for each.
Figure 18. Phosphorylation status of tAg.
Lysates of 3T3 cells which stably express the T proteins were subjected to IP with PAb 962. After washing, these lysates were resuspended in λ protein phosphatase buffer with or without λ protein phosphatase added. Lysates were incubated at 30° C for 30 minutes, and after washing, electrophoresed on an 18% polyacrylamide gel. Western blotting was performed using anti-T-protein antibodies.
CHAPTER 5
DISCUSSION
Roughly 80% of the global human population is seropositive for JC virus. Although the majority of individuals who are seropositive for JCV experience a lifelong asymptomatic infection, the clinical disease caused by JCV, PML, causes a fatal degenerative brain disease in up to 8% of AIDS patients, and is also observed in patients with other severe immunosuppressive disorders. In recent years JCV infection has been linked to several types of cancers including brain, colon and prostate cancer. Studies of SV40, the closely related prototype virus in this Polyomavirus group, have identified the viral early proteins as agents that activate specific signaling pathways involved in tumor progression including those that regulate the cell cycle. Although JCV and SV40 share approximately 70% sequence homology, they differ in their host and cell tropisms, levels of viral proteins expressed, association with specific cellular factors, and replication and transforming efficiencies. Many biological differences have been linked to differences in the functions of the tumor proteins of the two viruses. While a great deal of effort has been invested in the analysis of the SV40 TAg and tAg and JCV TAg, very little is known about the SV40 17KT and JCV tAg and T’ proteins. Clarifying the individual roles of JCV’s tumor proteins in transformation and in viral DNA replication will expand our understanding of how this virus interacts with host factors to abrogate cellular control of physiological processes.

5.1 Transformation assays

The initial goal of my thesis project was to compare the transforming potentials of JCV constructs expressing different combinations of viral early proteins using the dense focus assay (DFA). However, it became apparent that the sensitivity of this assay, as
performed previously in our laboratory, had decreased greatly; therefore it was readily apparent that my first step would be to restore the sensitivity of the DFA before using it to explore the contributions of the JCV early proteins in transformation. The DFA measures the ability of transformed cells to proliferate in medium supplemented with low concentrations of serum, overcome contact inhibition, and generate dense foci. In addition, cells which form foci during the course of an experiment can readily be isolated, tested for viral protein expression, and propagated for further analysis.

In past experiments performed by our group, SR:T+/t+/T'+ DNA has been used as a positive control in this assay. In this chimeric DNA construct, expression of wild-type JCV early proteins is driven by SV40’s promoter/enhancer signals. The more powerful SV40 transcriptional control sequences decrease the time required for foci to form from ~8 weeks using JCV DNA, to ~4-5 weeks. Rat2 cells, our standard cell line for this assay, transfected with this DNA typically exhibited ~30-40 foci per plate. We predicted that if fewer foci were induced by wild type DNA constructs, it would be difficult to measure a significant reduction in transforming activity by JCV early region mutants. Unfortunately, although I tested seven cell types, six transfection reagents, and six different transfection protocols, I was unable to induce more than approximately 20 foci per plate in any experiment. The single exception was an experiment using C3H10T1/2 cells which yielded higher numbers of foci but also showed a high background level. Further, I was unable to reproduce these numbers of foci in subsequent experiments.

It is possible that a poorer quality of fetal bovine serum now available is responsible for the reduced transforming efficiency currently measured in our DFA. It is also possible that the cell lines used for our transformation assays exhibit greater
resistance to viral transforming activity, due to repeated passaging and selection for cell variants. Transfection of SV40 DNA into the cells still results in the appearance of several hundred transformed foci per dish, but this number is reduced relative to earlier studies in which greater than a thousand foci were detected per plate (18, 69). The drop in transformation efficiency is even more apparent when testing constructs that express JCV proteins known to be much less robust than their SV40 counterparts.

In the Ras cooperation assay, DNAs encoding a combination of JCV early proteins are transfected into primary REF cells, with Ras expressed from a co-transfected plasmid which codes for the constitutively expressed protein. This assay examines the ability of Ras plus viral proteins to “cooperate” to overcome cellular senescence, drive proliferation in low serum, and induce a transformed phenotype and immortalization. Like the DFA, cells transformed by Ras plus viral proteins can readily be isolated and propagated for further study. Although I did isolate cell lines expressing various combinations of viral proteins (Figure 6), this assay does not yield reliable quantitative comparisons of transforming efficiencies of different combinations of tumor proteins.

I concluded that with the reagents and models we have available to us, performing the dense focus assay or variations of it is not a productive way to measure the transforming ability of JCV DNA, or more specifically, the contributions of different JCV tumor proteins to oncogenic potential in vitro. Still, the significant effort invested in generating a large collection of DNA clones expressing various combinations of JCV regulatory proteins proved useful for viral DNA replication and protein-protein interaction studies performed in my second project; they will also prove to be valuable reagents to our laboratory and the JCV field in the future.
5.2 tAg contributes to viral DNA replication.

JCV TAg plays many roles in initiation and elongation of viral DNA replication, and is critical to replication in permissive cells. A cell-free in vitro system of replicating plasmid DNA containing the JCV origin of replication, which was dependent on the addition of TAg alone, was developed in 1997 by Nesper et al. (110); in this study, replication was completely dependent on addition of TAg to a mix of three purified cellular proteins—polymerase α-primase, RPA, and topoisomerase I—or a crude lysate of human cells. In addition, specific residues of TAg are critical for its ability to facilitate initiation of replication. Alteration of Thr to Asp at residue 125, a phosphorylation site, abolishes replication (180). The T’ proteins complement TAg to mediate replication; the loss of any one or, in some cases, two of the T’ proteins has little effect on DNA replication, whereas the deletion of all three reduces replication 10- to 20-fold (123). The role of tAg in viral DNA replication has only lately begun to be addressed, although a large body of work performed on SV40 tAg suggests interesting initial experiments. In a recent report, a JCV genome containing a truncated tAg which encodes amino acids 1-89 exhibited reduced viral gene expression and viral DNA replication (148).

In this current study, I performed DpnI replication assays, which measure how effectively viral proteins expressed from DNA transfected into PHFG cells drive JCV DNA replication. Constructs expressing subsets of wild-type early proteins, as well as truncated or point mutant TAg and tAg proteins, were tested in these experiments. When all JCV proteins are expressed in PHFG cells, replication of the viral genome is readily detected by day 7. When TAg is the only early protein expressed, no replication at any time point is seen; this is in contrast to studies with SV40, in which TAg is both
necessary and sufficient for replication to occur (27). JCV TAg expression is powered by less robust promoter/enhancer signals and interacts with cellular proteins less efficiently than SV40 TAg, so it is possible that JCV TAg requires more cooperation from the other early proteins to drive viral DNA replication than SV40 TAg does. No JCV DNA replication was seen in cells expressing a truncated, unstable TAg in addition to wild type TAg and T’ proteins, indicating that TAg is necessary—although not sufficient—for JCV DNA replication. Expression of TAg alone results in no detectable viral DNA replication; however, when all JCV proteins other than TAg are produced in PHFG cells, viral DNA replication was also abolished. This suggests that although TAg alone is not sufficient to drive viral DNA replication, TAg plays a critical role in mediating viral DNA replication through cooperation with other early proteins in vivo. The requirement for TAg in replication may be due to its interaction with PP2A and effects on the downstream substrates of PP2A, since down regulation of PP2A reduces JCV DNA replication (148). TAg’s contribution to viral DNA replication may also occur through its interactions with the tumor suppressor proteins p107 and p130, which could lead to liberation of members of the E2F family of transcription factors. When TAg expression is abolished and an unstable truncated TAg is produced in addition to the three wild type T’ proteins, no replication is observed, as expected, since TAg is predicted to be necessary for replication.

I repeated the DpnI replication assay, and included three additional DNAs. Two of the DNAs express mutant TAg proteins—the P99A and C157A TAg mutants—and the third, a T’ deletion mutant, expresses wild type TAg and TAg but no T’ proteins. This T’ deletion mutant has been shown to replicate with 5-10% of the efficiency of wild type
JCV DNA. The role of the T’ proteins in viral DNA replication is unknown, but they may participate in sending cells to S phase via binding of Rb proteins. Inclusion of this DNA showed that the assay is capable of detecting DNA replication at levels 10- to 20- fold below that seen with wild type JCV DNA. Replication of the P99A and C157A tAg mutants was decreased 25- and 3-fold, respectively, compared to wild type JCV DNA, suggesting that these residues of tAg contribute to tAg’s ability to mediate viral DNA replication. The P99A mutation of tAg may alter tAg’s effect on the three-dimensional structure of the A subunit of PP2A, which may in turn alter tAg-mediated modulation of PP2A’s activity towards its cellular and viral substrates, while the C157A mutation of tAg may alter tAg’s interaction with members of the Rb family.

The J domain of SV40 tAg, which is common to all three SV40 early proteins, is composed of three α-helices, while the unique region is composed of four α-helices and two zinc-binding motifs, the first coordinated by a cluster of four cysteines (Cys103, Cys111, Cys113, and Cys116), and the second by a cluster of three cysteine and one histidine (His122, Cys138, Cys140, and Cys143). These zinc-binding motifs are distinct from the previously suggested GAL4-type zinc structure, and also from classic zinc-binding fingers (36) (Fig. 19). Both the N- and C-termini of SV40 tAg are located at the interface between the J and unique domains, and the N-terminus directly interacts with the A subunit of PP2A (36). The three dimensional conformation of JCV tAg is predicted to be similar to that of SV40 tAg, based on amino acid sequence similarity. Residue 99 of JCV tAg was found in this study to not be involved in binding of PP2A, p107 or p130; and mutation of this residue does not affect tAg stability. However, P99 is in the middle
Figure 19. The structure of SV40 tAg. The peptide chain is color-coded from blue to red, going through the rainbow colors, from the N terminus (blue) to the C terminus (red). The two zinc atoms are bound by cysteine clusters located in the unique region of tAg. Modified from Cho et al. (36).
of α-helix 4, which upon examination of the 3D protein conformation, is very close to the first zinc-binding domain (36) (Fig. 19). The role of JCV tAg’s zinc-binding motifs is unknown, but zinc-binding motifs in SV40 TAg are required for hexamer formation, initiation of viral DNA replication, and protein stability (98), and it is possible that tAg’s zinc-binding motifs are also involved in viral DNA replication.

C157A was also found to not be involved in binding of PP2A, p107 or p130, or in tAg stability. It is located in the middle of α-helix 7, which is not particularly close to either zinc-binding domain or to the J domain interface. The C157 residue of tAg appears to contribute to tAg-mediated viral DNA replication by an unknown mechanism.

In an attempt to measure the effect of the H24Q mutation on tAg’s contribution to JCV DNA replication, I co-transfected PHFG cells with one plasmid expressing the H42Q tAg, and second expressing wild type TAg and T’ proteins. Since the H42 residue is shared by all five early proteins, a mutation in residue H42 of tAg would also affect TAg and the T’ proteins; therefore I generated a construct which expressed the mutant H42Q tAg alone, and used it in conjunction with a tAg deletion construct for this experiment. I also co-transfected PHFG cells with a plasmid expressing wild type tAg and another expressing TAg and the T’ proteins, as a positive control. Neither the cells expressing the H42Q tAg nor those with wild-type tAg exhibited any detectable DNA replication, and therefore no conclusions could be reached regarding the effect of the H42Q tAg mutation on viral DNA replication potential. The data did initially suggest that the tAg coding region must be cis to the origin of viral DNA replication. However, in a study examining the role of SV40 tAg in replication, addition of tAg protein to cells infected with SV40 tAg-defective genomes stimulated replication (38). This suggests that
although the presence of tAg stimulates efficient viral DNA replication, the coding sequence itself is not required, at least in the case of SV40.

The SV40 and mPyV TAgs have long been recognized as the key replication protein of these viruses, and in vivo and in vitro studies have confirmed this role for JCV TAg as well. A cell-free DNA replication system utilizing SV40 TAg indicated that addition of PP2A to the cell-free system enhanced DNA replication and that SV40 tAg inhibited this effect (92). However, SV40 tAg appeared to stimulate SV40 DNA replication to a limited extent in vivo; SV40 tAg deletion mutants produced slightly lower burst sizes of viral progeny (175). Recently, a truncated JCV tAg mutant, encoding amino acids 1-89, was shown to replicate with similar efficiency to wild type JCV at days 7 and 14 p.t., but significant inhibition was observed at the day 21 time point (54). The authors speculated that reduced replication resulted from the inability of the mutant tAg to inhibit PP2A, leading to dephosphorylation of agnoprotein, a late JCV regulatory protein, that as a phosphoprotein contributes to efficient DNA replication.

By modifying the replication assay and increasing expression of the JCV early proteins under the control of more potent CMV transcriptional signals, low levels of JCV DNA replication were observed; a construct lacking tAg and the three T' proteins still failed to replicate. With high levels of viral proteins from the heterologous CMV transcription signals, low but detectable levels of replication of tAg-deficient JCV DNA can be demonstrated. The requirement for tAg in viral DNA replication is not absolute in this system. Promoter/enhancer signals affect many aspects of viral DNA transcription and translation, including recruitment of cellular transcription machinery to viral DNA, alternative splicing of early viral mRNA, and transcripts levels of viral protein.
expression, and it is possible that the CMV promoter sequence altered the dynamic of the interactions between viral proteins and the cellular environment. These data indicate that JCV tAg, unlike its SV40 counterpart, makes a significant contribution to viral DNA replication activity, and furthermore, that its effect on agnoprotein phosphorylation status does not explain this role in replication, since agnoprotein is not present in the assay.

5.3 tAg interacts with PP2A.

JCV tAg shares 79% sequence homology to SV40 tAg. However, while the N-terminal sequences (residues 1-81; JCV) of these proteins are highly conserved with 82% homology, there is more variability in the C-terminal areas (residues 82-172) of these proteins; the unique regions of SV40 and JCV tAgs share only 54% homology. The degree of sequence similarity suggests that while some functional overlap exists, the tAg proteins of different polyomaviruses are likely to make unique contributions to virus-induced cellular transformation or viral replication.

In a recent study, a GST-tAg fusion protein synthesized in bacterial cells was found to interact with PP2A; this interaction lead to a modification of PP2A’s dephosphorylating abilities (148). Agnoprotein, one of JCV’s late proteins, was found to be a target of PP2A; when tAg interacts with PP2A, the dephosphorylation of Agnoprotein by PP2A is inhibited (148). Down regulation of PP2A was found to cause significant reduction in JCV replication (148), which suggests that tAg’s effects on PP2A’s activity plays an important role in viral infection. In a recent study, it was hypothesized that reduction of viral DNA replication in cells transfected with DNA encoding a truncated tAg mutant was due to reduction of tAg-mediated PP2A inhibition,
leading to dephosphorylation of agnoprotein. Phosphorylation mutants of agnoprotein have been shown to be defective for viral DNA replication (147). However, in the experiment in which I co-transfected DNAs encoding wild type early proteins, a tAg deletion mutant, or TAg alone under the control of a CMV promoter in addition to a plasmid containing the JCV origin of replication, agnoprotein was not present; but expression of tAg stimulated DNA replication substantially. The effect of tAg-mediated PP2A inhibition on agnoprotein may contribute to viral DNA replication, but it is not the only contribution of tAg to replication.

Bacterially synthesized GST-JCV tAg was previously found to bind to the AC core of the PP2A holoenzyme in vitro, and amino acids 82-124 of tAg are critical to this interaction (148). Here, I verified through co-IP/WB experiments that tAg interacts with the C subunit of PP2A in vivo, in REF and 3T3 cells. PP2A is present in cells as either a trimeric holoenzyme consisting of the AC core plus a regulatory B subunit, or as the AC core by itself. The A and C subunits are not found as free proteins at high levels (139), and SV40 tAg does not bind to purified free C subunit (198); this suggests that JCV tAg also binds the AC core, rather than the C subunit directly.

The proline at residue 99 in the JCV tAg is conserved in SV40, mPyV, and BKV proteins, and this amino acid is critical to PP2A binding by SV40 tAg. The conserved cysteine at residue 157 in the JCV tAg is part of the second of two recently recognized LxCxE motifs. LxCxE motifs in TAg and the T’ proteins are required for binding of the Rb proteins and, in cooperation with the J domain, release of the E2F family of transcription factors. Neither alteration of the JCV tAg proline 99 to alanine, or cysteine 157 to alanine, detectably reduces binding to PP2A. Binding of tAg to PP2A and
subsequent alteration of its specificity and activity are likely important for JCV tAg’s contributions to viral DNA replication abilities. Neither P99 nor C157 are critical to the binding between JCV tAg and PP2A. However, the scaffolding A subunit of PP2A undergoes a pronounced conformational change when a regulatory B subunit is added to the core AC enzyme. This flexibility of the A subunit is hypothesized to play a role in the holoenzyme’s function (196). The P99A or the C157A mutations might alter the tAg-PP2A interaction in such a way as to affect the conformational changes the A subunit undergoes when the viral protein binds the AC core in place of a B subunit. The altered conformation of the AC core-tAg complex could affect the affinity of the complex for one or more of its substrates. Proline 99 is structurally located very close to the first zinc-binding motif (36) (Fig. 6), which is required for binding of SV40 tAg to PP2A. It is possible that when this amino acid is mutated, it changes tAg’s three dimensional conformation in such a way that its interaction with PP2A results in PP2A activity and specificity more like that of the normal ABC PP2A holoenzyme rather than AC-tAg. This would explain why although the P99A and C157A tAg mutants appear to bind PP2A as strongly as wild type tAg, constructs expressing both of these tAg mutants exhibit replication defects.

5.4 tAg interacts with the Retinoblastoma proteins p107 and p130.

The LxCxE motif in TAg and the T’ proteins are predicted to cooperate with the J domain to effect the release of members of the E2F family of transcription factors from Rb proteins, thus affecting cell cycle progression. The two recently recognized LxCxE motifs of tAg may play a similar role in the viral induction of cellular proliferation.
Lysates prepared from cell lines expressing wild type tAg or the P99A or C157A mutant tAgs were subjected to co-IP/WBs to determine whether tAg bound p107 or p130. The wild type and both mutant tAgs interacted with both p107 and p130; no detectable reduction in binding was observed between the mutant tAgs and these cellular proteins. The C157A mutation in tAg alters the central cysteine residue in the motif, similar to a mutation in the LxCxE of TAg which disrupts the interaction between TAg and the Rb proteins. Disruption of the second motif does not affect interaction with the Rb proteins. This suggests that binding of tAg with p107 and p130 may occur through the first LxCxE motif, or that the two motifs are redundant and that one functional motif is capable of facilitating binding. Since tAg has a J domain as well as LxCxE motifs, the two domains may cooperate to mediate viral regulation of cell cycle progression. Interaction of tAg with the tumor suppressor proteins p107 and p130, and release of members of the E2F family transcription factors normally bound to them, may be one mechanism by which tAg influences viral DNA replication.

### 5.5 tAg is a phosphoprotein

An extensive body of literature documents the influence of phosphorylation upon the regulation of polyomavirus TAg functions [reviewed in (143)]. The JCV TAg contains two major phosphorylation domains, composed of threonine and serine residues clustered at the N and C termini (170). The JCV T’135, T’136 and T’165 proteins also share TAg’s N-terminal phosphorylation domain, and modification of at least one site, threonine 125, is regulated, in part, by cell cycle mechanisms (19). We noted that in several of our IP/WB experiments that JCV tAg migrated as either one to three bands on
the gels. Lysates of cells expressing all five wild-type JCV early proteins, or tAg alone, were treated with λ protein phosphatase. These and untreated lysates were subjected to IP/WB to examine viral early proteins. The slowest migrating of the multiple bands representing each T' as well as tAg in the untreated samples were not present in the treated sample. It is possible that the multiple bands represent differentially phosphorylated tAg species. The software program PredPhospho (83) predicts four potential phosphorylation sites in the JCV tAg; at serine 10, threonine 92, threonine 165, and tyrosine 167. Studies examining the potential modification of these or other sites in JCV tAg are now ongoing.

JCV tAg possesses several unique features among polyomavirus tAg proteins. Unlike SV40 tAg, it plays a central role in replication of viral DNA. SV40 tAg has been found to interact with PP2A and it is predicted that it binds Hsc70; in addition to these two cellular proteins, JCV tAg binds the viral agnoprotein and the Rb proteins, p107 and p130, via its two unique LxCxE motifs. These motifs may cooperate with its J domain to alter cell cycle progression. Mutation of the conserved proline at position 99 in JCV tAg does not lead to detectable loss of binding to PP2A, while the same mutation in SV40 tAg results in a substantial binding defect. Finally, preliminary evidence suggests that the JCV tAg is modified by phosphorylation, although we have not found reports in the literature suggesting this to be true for the SV40 protein. The study of JCV tAg has only recently begun, and it is expected that a greater understanding of this viral tumor protein will provide new insights into the pathogenic and oncogenic potentials of this human virus.
CHAPTER 6

FUTURE EXPERIMENTS
Hypothesis: The two zinc-binding motifs in the unique C-terminal sequence of tAg are required for binding of PP2A.

Rationale: The N-terminal 81-amino acid sequence of tAg is shared with TAg and the three T' proteins, but tAg is the only early JCV protein which binds to PP2A, suggesting that the PP2A-binding region is located in its unique C-terminal 91 amino acids. Additionally, the unique region of the closely related SV40 tAg is known to be the region responsible for binding to PP2A.

Experiment: A series of deletion mutants of tAg could be generated to identify amino acid residues that influence PP2A binding. A wild-type JCV DNA template will be subjected to a site-directed mutagenesis PCR protocol, to alter nucleotides to code for a stop codon instead of an amino acid residue. Each point mutant will be tested for protein stability, and binding to PP2A, via co-IP/WB analysis. This will elucidate which regions of tAg are required for binding with PP2A. Once the region of tAg’s sequence required for PP2A binding has been narrowed, specific amino acid residues within that sequence can be targeted for point mutation to analyze their importance to PP2A binding. Lys126, Asp131, Pro132, Leu133, Met146, Trp147, Phe148, and Gly149 of SV40 tAg have been found to bind PP2A (35, 36). All but Asp131 and Met146 are conserved in JCV tAg; the six conserved residues plus Ser129 and Gln144, homologous to the two nonconserved residues, would be natural first choices for mutational analysis.

Hypothesis: The LxCxE sequences of tAg are important in binding p107 and/or p130.

Rationale: LxCxE motifs are found in cellular and viral proteins. These motifs bind members of the Rb family and can facilitate cell cycle progression by mediating release
of the E2F transcription factors bound the Rb proteins. tAg was shown in the current study to interact with p107 and p130. We have observed in the current study that disruption of the central cysteine in the second LxCxE motif with a C157A mutation does not inhibit binding of either p107 or p130, indicating that there may be some redundancy. tAg will be subjected to disruption of the first LxCxE motif, and both motifs together. These mutant tAgs can then be tested for binding to the Rb proteins.

**Experiment:** Point mutations of the LxCxE motifs of tAg, will be generated via a site-directed mutagenesis (SDM) PCR protocol. DNA encoding amino acid residues from each motif, both singly and together, will be mutated. These DNAs will be transfected into cells, and lysates of cell lines expressing each mutant tAg will be analyzed via co-IP/WB for binding to p107 and p130 and compared to binding between TAg and the Rb proteins, and the T' proteins and the Rb proteins. I predict that reduced binding of tAg with the Rb proteins will be detected in lysates from cells expressing tAg with mutated LxCxE motifs.

**Hypothesis:** One or both of tAg’s LxCxE domains cooperate with the J domain to influence the phosphorylation status of p107 and/or p130.

**Rationale:** An intact LxCxE domain is required for interaction of T proteins with pRb, p107 and p130. The J domain contained in the first 81 amino acids which are common to all five early proteins are also required for TAg to affect the phosphorylation status of p130 (164, 180). JCV tAg contains both an LxCxE and a J domain; these regions may cooperate to allow tAg to bind Rb proteins, which could lead to a change in their phosphorylation status through recruitment of PP2A. Disruption of either the LxCxE
sequence or the J domain may reduce or abolish tAg-mediated dephosphorylation of the Rb proteins.

**Experiment:** Lysates of cells which express no viral proteins, wild type tAg, single or double LxCxE mutant tAgs, or a J domain mutant tAg, will be subjected to IP/WB analysis to detect the presence and interaction of p107 and p130. The phosphorylation status of p107 and p130 will be examined in these lysates. I predict that lower levels of phosphorylated Rb proteins will be present in cells expressing wild type tAg than in cells expressing no viral protein, tAg with a double LxCxE mutation, or the J domain mutant tAg.

**Hypothesis:** JCV tAg functions are regulated, in part, by phosphorylation.  
**Rationale:** The collective phosphorylation status of a protein’s potential phosphorylation sites has the potential to regulate protein function both positively and negatively.  
**Experiment:** Four potential phosphorylation sites are predicted by PredPhospho software (86): Ser10, Thr92, Thr165 and Tyr167. A series of tAg point mutants could be generated to abolish modification at each predicted phosphorylation site. The stability of each mutant protein would be examined via IP/WB of lysates prepared from cells transfected with these DNAs. Viral DNA replication of each stable mutant could then be tested to determine which modified sites might be critical to tAg’s ability to mediate DNA replication.
REFERENCES


SV40 small t antigen form stable complexes with protein phosphatase 2A. Cell 60:167-76.


APPENDIX: THE CONSTRUCTION OF RECOMBINANT DNAs EXPRESSING VIRAL EARLY PROTEINS
Figure 1. pSR:muT+/t+/T'+
A. pJR:T+/t+/T'+ was digested with AflII, which cuts twice within the TAg coding region to excise a 734 bp fragment. The backbone was gel-isolated and self-ligated.
B. pJR:muT+/t+/T'+ was then digested with NcoI, which cuts twice at sites flanking the 425 bp JCV regulatory region (JR). This backbone was gel-isolated and purified. The homologous SV40 regulatory region (SR) 427 bp fragment (digested with NcoI and gel-purified from pSR:T+/t+/T'+) was ligated to this backbone to produce pSR:muT+/t+/T'+, the final product. The orientation of the SRR fragment was determined by an AvrII/BamHI digest, and then confirmed by sequencing.
A. tAg intron removed: 4494/4426

EcoNI site
nt 4877

nt 4605: A→G
in mutant
tAg cDNA

nt 4605: A→G
(in mutant tAg cDNA)

PfMI site
nt 4413

pCR 2.1 TA cloning vector

tAg intron removed: 4494/4426

EcoNI

PfMI

ptCR2.1-small tAg
or
ptCR2.1-small tAg clone2 (mutant)

B. tAg intron removed: 4494/4426

EcoNI site
nt 4877

nt 4605: A→G
(mutations in
tAg cDNA)

PfMI site
nt 4413

Ligation into
vector backbones

723 of pCR3/
5019 of JCV

CMV:T+/t-/T'+
backbone

CMV vector
(pCR3, Invitrogen)

nt 4605: A→G
2473 of JCV/20 bp of adapter
sequence, then
nt 759 of pCR3

EcoNI

PfMI

723 of pCR3/
5019 of JCV

CMV:T-/mut+/T-'
C. (This CMV construct containing mutant tAg cDNA was also used to generate a second construct, pJR:T-/mut+/T-.)

CMV:T-/mut+/T-

4494/4426

723 of pCRII/
5019 of JCV

5019 of JCV

m 4605:
A → G

2473 of JCV:
20 bp of adapter sequence, then cut 759 of pCR3

CMV:T-/t+/T-

4494/4426

723 of pCRII/
5019 of JCV

m 4605:
wild-type

2473 of JCV:
20 bp of adapter sequence, then cut 759 of pCR3

D. 723 of pCRII/
5019 of JCV

m 4605:
wild-type

Removed tAg exon 1
(4494,4426)

4274: wild-type

 donor splice site

<--1196--> BstXI 4615

PstI 3355

Ligation of wild-type tAg cDNA fragment into CMV backbone to produce CMV:T+A+/T-.

CMV:T+/t-/T+ backbone

129
E. Fragment sizes seen on a gel:
- 5653 bp
- 1196 bp
- 418 bp

F. Ligation of tAg cDNA fragment into pJR:T+/t+/T+ backbone to create pJR:T-+T-. 

Removed taq exon 1 (4494-4426)
Figure 2. Construction of pSR:T-/t+/T'-.

A. PCR was performed on CMV-T+/t+/T'+ using tAg cDNA F and tAg cDNA R primers to amplify tAg cDNA. Primer tAgcDNA F anneals at nucleotide (nt) 4980-4942. The other primer, tAgcDNA R, has a “tail” that does not anneal which is composed of nt 4413-4426; the remainder of the primer consists of the JCV sequence from nt 4494-4517 and anneals to the template at those nts. The tAg cDNA PCR product generated consists of 19 nt which compose the “tail” of the R primer. The first 5 nts are not composed of JCV sequence—they are present to increase the efficiency at which the PflMI site at nt 4413-4423 is cleaved—and the remaining 14 nts consist of nt 4413-4426. The tAg intron from nt 4427-4493 is not present. Two PCR products, wild-type tAg cDNA and a second clone (an artifact of the PCR process), a tAg cDNA 1-bp mutant, were isolated and sequenced, and ligated into the linear TA cloning pCR2.1 vector.

B. The tAg cDNA fragments were digested with EcoNI and PflMI, gel purified, and ligated into a CMV:T+/t-/T'+ backbone in a pCR3 vector (CMV:T+/t-/T'+ was digested with EcoNI and PflMI and the backbone was gel-purified). When the tAg intron is removed, neither TAg nor the T' proteins are expected to be produced since the acceptor splice site for TAg's and the T' proteins' common first intron has then been joined to tAg's donor splice site and is expected to be unuseable. CMV:T-/mut+/T'-, which has the 1 bp mutant tAg sequence and the wild-type T' donor splice site was generated.

C. Site-directed mutagenesis was employed to generate a construct with the wild-type TAg sequence in the wild-type backbone. CMV:T-/mut+/T'- was used as a template in a PCR reaction with primers WTtAgE and WTtAgL, which consist of complementary wild-type tAg cDNA sequence from nt 4591-4628.

D. The region of interest, which contains the wild-type TAg sequence as well as the wild type T' donor splice site region, was digested out with BstXI and PstI. It was then ligated into a CMV backbone that had not undergone PCR amplification with Pfu.

E. CMV:T-/t+/T'- and pJR:T+/t+/T'+ were both digested with both BstXI and PstI. The fragments highlighted in red were gel-isolated and purified, then ligated together in a 3-fragment ligation to create pJR:T-/t+/T'-.

F. The orientation of the fragments in the ligation product was screened via a BamHI/EcoNI digest.

G. pJR:T-/t+/T'- was then digested with NcoI, which cuts twice at sites flanking the 425 bp JCV regulatory region (JR). The backbone was gel-isolated and purified. The homologous SV40 regulatory region (SR) 427 bp fragment (digested and gel-purified with NcoI from pJR:T+/t+/T'+ ) was ligated into this backbone to produce pSR:T-/t+/T'-.

The orientation of the SRR fragment was screened via a AvrII/BamHI digest, and confirmed by sequencing.
A. Primers used in PCR reaction to alter mutant tAg sequence to wild type:

- WTtAgE and WTtAgL

- T donor splice site mutated: nt 4274

- tAg intron removed: 4494/4426

B. Wild-type tAg sequence at nt 4605

- T donor splice site mutated: nt 4274

- BglII 4242

- 1722 of JCV 4361 of pBR322

- 7212

pSR: T-/+T'/t'
Figure 3. Construction of pSR:T-/t+/T'd-.
A. Site-directed mutagenesis was used to alter pSR:T-/mut+/T'd- to pSR:T-/t+/T'd-. The template, pSR:T-/mut+/T'd-, was amplified and altered in a PCR reaction using complementary primers called WTtAgE and WTtAgL, which span the wild-type tAg coding sequence from nt 4591-4628. The PCR product, after DpnI digestion and visualization on a gel, was transformed into competent cells. After screening plasmid DNA purified from colonies of competent cells via restriction enzyme analysis, the region of DNA between BstXI and BglII (nt 4615 and 4242, respectively) was sequenced for confirmation.
B. The sequenced, correct pSR:T-/t+/T'd- clone was then digested with BstXI and BglII, to sequester the sequenced region including the wild-type tAg cDNA sequence (with the tAg intron removed), and the mutated T' donor splice site. The 305 bp fragment encompassing these areas was gel-isolated. pSR:T-/mut+/T'd- was also digested with BstXI and BglII and the two fragments which make up the plasmid backbone, the 1905 bp and 7212 bp fragments, were gel-isolated. These two fragments from pSR:T-/mut+/T'd- a were ligated together with the 305 bp fragment from pSR:T-/t+/T'd- to generate a clone of pSR:T-/t+/T'd- with the sequenced area of interest in a plasmid backbone (that was not generated via PCR, as the backbone of the original pSR:T-/t+/T'd- clone was). The orientation of the fragments was screened via a BamHI/EcoNI digest.
The following product was generated:
CMV-TAg-mutsmalltAg (CMV:T+/mut+/T-)
Has 1 bp mutant tAg and the wild-type T donor splice site.
C.

Fragment sizes seen on a gel:
- 5653 bp
- 1196 bp
- 418 bp

D.

1721 of JCV
1 of pBR322

removed

1/5130 of JCV

1722 of JCV
4361 of pBR322

PstI

PstI

1721 of JCV
1 of pBR322

PstI

PstI

4615

4615

PstI

PstI

3355

3355

PstI

PstI

1/5130 of JCV

1722 of JCV
4361 of pBR322

PstI

PstI

3607 of pBR322

PstI

PstI
E.

1721 of JCV 1 of pBR322
333
Neot
275

5136
Neot
4980
At 4605: A→G

1722 of JCV 4361 of pBR322

pJR:T- /mut+/ T'- 9423 bp each

1721 of JCV 1 of pBR322
275 (333)
Neot

4494/4426
Neot
(5136) 4980
At 4605: A→G

1722 of JCV 4361 of pBR322

pSR:T- /mut+/ T'- 9425 bp each
Figure 4. Construction of pSR:T-/mut+/T'-.
A. PCR was performed on CMV-JCVE using tAg cDNA F and tAg cDNA R primers to amplify tAg cDNA. A 1-bp tAg cDNA mutant PCR product was isolated and sequenced, and ligated into the linear TA cloning pCR2.1 vector.
B. The tAg cDNA fragment was digested with EcoNI and PflMI and gel purified. CMV:T+/t-/T'+ (in pCR3) was digested with EcoNI and PflMI to remove the corresponding tAg coding region, and the backbone was gel-purified. The EcoNI-PflMI tAg fragment was then ligated into the CMV:T+/t-/T'+ backbone.
C. The CMV-mutant tAg construct and pJR:T+/t+/T'+ were both digested with both BstXI and PstI. The fragments highlighted in red were gel-isolated and purified, then ligated together in a 3-fragment ligation to create pJR:T-/mt+/T'-.  
D. After ligation of mutant tAg cDNA fragments to a pJR:T+/t+/T'+ backbone to create pJR:T-/mut+/T'-, the orientation of the fragments was screened via a AflII digest.
E. pJR:T-/mut+/T'- was then digested with NcoI, which cuts twice at sites flanking the 425 bp JCV regulatory region (JR). The backbones were gel-isolated and purified. The homologous SV40 regulatory region (SR) 427 bp fragment (digested and gel-purified with NcoI from pSR:T+/t+/T'+) was ligated into these backbones to produce pSR:T-/mut+/T' and pSR:T-/mut+/T'd-, the final products. The orientation of the SR fragment was screened via an AvrII/BamHI digest.
A. tAg intron removed: 4494/4426

EcoNI site nt 4877
PfMI site nt 4413

nt 4605: A→G
(in mutant tAg cDNA)

pCR2.1 TA cloning vector

B. tAg intron removed: 4494/4426

EcoNI site nt 4877
PfMI site nt 4413

nt 4605: A→G
(mutation in tAg cDNA)

CMV:T+/t-/T'Δ was generated, which has 1 bp mutant tAg, and the T' donor splice site mutation.
C.
Fragment sizes seen on a gel:
5653 bp
1196 bp
418 bp

D.
Removed tag exon 1 (4406-4426)
Ligation of mutant tag cDNA fragment into
pM7TCR1A backbone to create pJR:T+/mut+/T\''d.

Fragment sizes seen on a gel:
5843 bp
2382 bp
1265 bp
Figure 5. Construction of pSR:T-/mut+/T'd-.
A. PCR was performed on CMV:T+/t+/T'+ using tAg cDNA F and tAg cDNA R primers to amplify tAg cDNA. A 1-bp tAg cDNA mutant PCR product was isolated and sequenced, and ligated into the linear TA cloning pCR2.1 vector.
B. The tAg cDNA fragment was digested with EcoNI and PflMI and gel purified. CMV:T+/t-/T' (in pCR3) was digested with EcoNI and PflMI to remove the corresponding tAg coding region, and the backbone was gel-purified. The EcoNI-PflMI tAg fragment was then ligated into the CMV:T+/t-/T'- backbone.
C. The two CMV-mutant tAg constructs and pJR:T+/t+/T'+ were all digested with both BstXI and PstI. The fragments highlighted in red were gel-isolated and purified, then ligated together in a 3-fragment ligation to create 2 constructs: pJR:T-/mt+/T'-, and pJR:T-/mt+/T'd-.
D. After ligation and transformation, the orientation of the fragments was screened via a AflII digest.
E. pJR:T-/mut+/T’d- was then digested with NcoI, which cuts twice at sites flanking the 425 bp JCV regulatory region (JR). The backbone was gel-isolated and purified. The homologous SV40 regulatory region (SR) 427 bp fragment (digested and gel-purified with NcoI from pSR:T+/t+/T'+) was ligated into these backbones to produce pSR:T-/mut+/T'd-. The orientation of the SR fragment was screened via an AvrII/BamHI digest.
**A.**

Fragment sizes seen on a gel:
- 2783
- 2148
- 1284
- 697
- 373

**B.**

Fragment sizes seen on a gel:
- 5053
- 2382
- 1630
- 425

**Ligation of TAg coding fragment and SR fragment to pJR:T+/t+/T+ backbone to create pSR:T+/t+/T+.**
**Figure 6. Construction of pSR:T+/t-/T'+.**

A. CMV:T+/t-/T'+ and pJR:T+/t+/T'+ were both digested with NcoI and PstI. The fragments highlighted in red were gel-isolated and purified, then ligated together to create pJR:T+/t-/T'+. Instead of using the NcoI-NcoI 425 bp fragment containing the JCV regulatory region (JR), the 427 bp NcoI-NcoI SV40 regulatory region (SR) was gel-isolated from pJR:T+/t+/T'+ after NcoI digestion (not shown here), and used in this ligation.

B. A 4 fragment ligation was performed: 2 fragments from the digestion of pJR:T+/t+/T'+ with PstI and NcoI; 1 SRR fragment; and the TAg cDNA fragment from the digestion of CMV:T+/t-/T'+ is the fourth.

C. Since the PstI-PstI fragment and the NcoI-NcoI fragment each could insert in either orientation, pSR:T+/t-/T'+ clones were screened via KpnI/AflIII digest, which gives the following patterns. Once a correct clone was identified with this screen, the DNA was sequenced for verification.
Figure 7. Construction of pSR:muT+/t-/T'+

pSR:T+/t-/T'+ was digested with AflII, which cuts twice within the TAg coding region to excise a 734 bp fragment. The backbone was gel-isolated and self-ligated. Clones were screened via an AflII digest.
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