

**REGULATION OF THE HUMAN NEUROTROPIC POLYOMAVIRUS, JCV, IN  
THE CENTRAL NERVOUS SYSTEM**

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to the Temple University Graduate Board

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of the Requirements for the Degree  
DOCTOR OF PHILOSOPHY

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by  
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## **ABSTRACT**

Title: Regulation of the human neurotropic polyomavirus, JCV, in the CNS.

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The human neurotropic virus, JC virus (JCV), is the etiologic agent of the fatal demyelinating disease of the central nervous system, progressive multifocal leukoencephalopathy (PML) that is seen primarily in immunodeficient individuals. Productive infection of JCV occurs only in glial cells and this restriction is to a great extent due to the activation of the viral promoter that has cell type-specific characteristics. The cell types that support the JCV infection cycle in culture are limited to primary human fetal glial cells and several transformed cell lines of glial origin. We developed a new hybrid cell system permissive for JC virus infection in order to gain insight into the mechanisms responsible for cell type specificity of JCV. The new cell system was created through the use of polyethylene glycol (PEG)-mediated cell fusion of primary human fetal astrocytes (PHFA) with an HPRT-deficient glioblastoma cell line, U-87MG. The new hybrid system was then used to analyze the ability of JCV replication and gene expression by infection studies. Results demonstrated that the new hybrid lines efficiently support JCV propagation during the early passages but lost that property in later passages. Earlier studies led to the assumption that glial-specific activation of the JCV

promoter is mediated through the involvement of positive and negative transcription factors that control reactivation of the JCV genome under normal physiological conditions and suppress its activation in non-glial cells. Here we demonstrate that the alternative splicing factor, SF2/ASF, has the capacity to exert a negative effect on transcription of the JCV promoter in glial cells through direct association with a specific DNA sequence within the viral enhancer/promoter region. Our results show that down-regulation of SF2/ASF in fetal and adult glial cells increases the level of JCV gene expression and replication indicating that negative regulation of the JCV promoter by SF2/ASF may control reactivation of JCV replication in brain. JCV induces a broad range of neural-origin tumors in experimental animals has been repeatedly detected in several human cancer most notably neural-crest origin tumors, including medulloblastomas and glioblastomas. The oncogenic activity of JCV is attributed to the viral early gene products, large T and small t antigen, as evidenced by the results from in vitro cell culture and in vivo transgenic animal studies. We demonstrate that SF2/ASF suppresses the expression of large T antigen and small t antigen in JCV-transformed tumor cell lines. Expression of SF2/ASF in such tumor cells ultimately hinders the transforming capacity of the viral tumor antigens. Moreover, downregulation of SF2/ASF in viral-transformed tumor cell lines induces the growth and proliferation rate of the tumor cells.

Altogether, we have created a new hybrid cell system which may serve as a good model system to study the biology of JCV aimed at identifying cellular determinants of the virus replication and gene expression as well as developing

novel therapeutic intervention strategies against JCV-induced disease, PML. We have also demonstrated a novel role of the cellular alternative splicing factor, SF2/ASF, in the regulation of JCV gene expression and transformation. These observations provide a new avenue of research to understand pathogenesis of JCV-induced diseases through interplay between JCV regulatory proteins and host factors, such as SF2/ASF.

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# CHAPTER 1

## INTRODUCTION

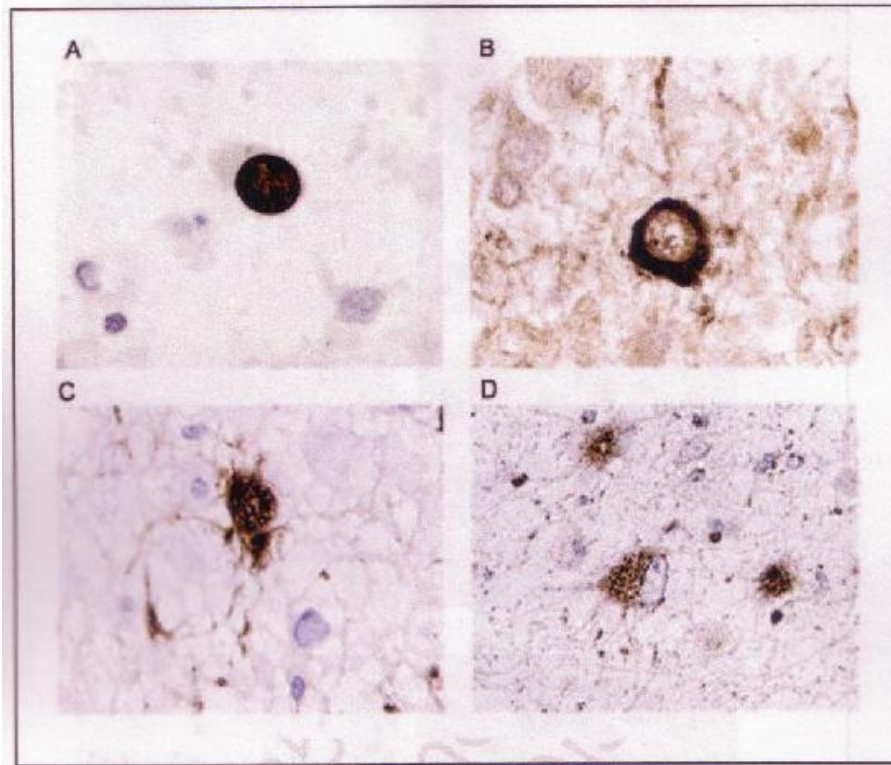
### 1.1 JC VIRUS AND PML

JCV is a neurotropic virus that lytically infects oligodendrocytes in the central nervous system and causes a neurodegenerative disease of the white matter in the human brain, progressive multifocal encephalopathy (PML). The disease develops mostly in patients with underlying immunosuppressive conditions, including Hodgkin's lymphoma, lymphoproliferative diseases, and AIDS (Major, 1992; Berger and Concha, 1995; Berger and Major, 1999). In a small number of cases, however, PML was also found to affect individuals with no underlying disease (Major, 1992; Berger and Concha, 1995). While PML was previously considered a rare complication in middle-aged and elderly patients with lymphoproliferative diseases, due to the AIDS epidemic in recent years, it is now a disease of the CNS equally encountered in patients of different age groups. This suggests that human immunodeficiency virus (HIV) infection may directly or indirectly participate in this process. Recent estimates indicate that the incidence of PML in HIV-seropositive patients reached up to 5%, compared to that 0.8% before the AIDS epidemic (Aksamit et al, 1990; Aksamit, 1995; Berger and Concha, 1995; Berger et al, 2001).

Recently PML has been described in patients with autoimmune diseases treated with immunomodulatory therapies. The monoclonal antibodies, natalizumab and efalizumab, are examples of these biological therapies.

Natalizumab and efalizumab bind to alpha-integrin molecules on the surface of B and T cells, and prevent their entry into the brain. Another example is rituximab which binds to the CD20 molecule on the surface of B cells, causing their depletion from peripheral circulation by activating the complement cascade. During the clinical trial of Tysabri (natalizumab), PML has been diagnosed in two multiple sclerosis patients and in one Crohn's patient in 2005 (Sandborn et al., 2005; Langer-Gould et. al., 2005; Kleinschmidt-DeMasters and Tyler, 2005). PML has been detected in four plaque-psoriasis patients treated with Raptiva (efalizumab). PML has also been detected in approximately 57 patients treated with Rituxan (rituximab) used in lymphoma patients and some rheumatoid arthritis patients (Carson et. al., 2009). Currently, there is no treatment for PML.

Clinically, PML patients develop subcortical dementia, visual disturbances, and hemiplegia. Magnetic resonance imaging (MRI) reveals multifocal demyelination mostly in white matter and some cases in cerebral hemispheres. Histopathological findings of PML are multifocal myelin loss, enlarged oligodendroglial nuclei, and enlarged bizarre astrocytes with lobular nuclei. JC virus proteins can be detected in infected oligodendrocytes and astrocytes by immunohistochemical analyses (Figure 1.1).



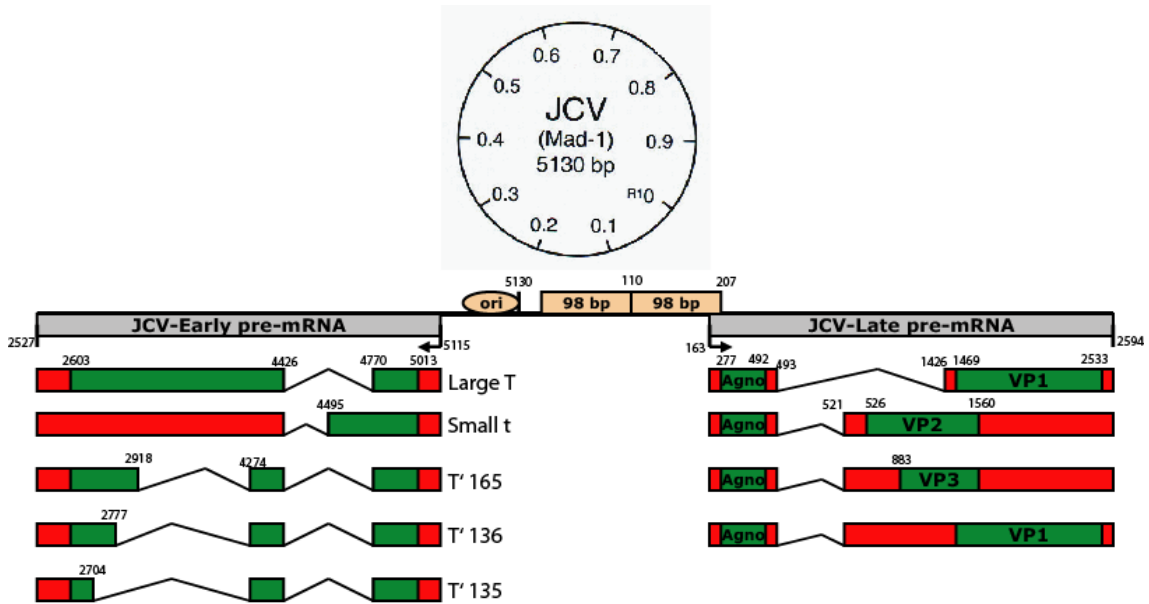
**Figure 1.1: Immunohistochemical analyses of PML lesions for the presence of JC viral proteins.** VP1 capsid protein is localized to the nucleus while agnoprotein is observed in the perinuclear and cytoplasmic regions of inclusion bodies within infected oligodendrocytes (A and B, respectively). In bizarre astrocytes, VP1 and agnoprotein are both detected in the nucleus and the cytoplasm (C and D, respectively). (Reproduced from reference 27, with permission from Landes Bioscience and Springer Science+Business Media).

JCV was first isolated from brain tissue of a PML patient by Padgett et al., in 1971. The brain tissue was used as a source of inoculum to infect primary cultures derived from human fetal brain, and the virus was successfully isolated from long-term cultures mainly consisting of glial cells (Padgett, 1971). This was

the first direct evidence suggesting that a neurotropic viral agent was associated with the occurrence of PML. Shortly after its isolation, the oncogenic potential of the virus was tested both in tissue culture and in experimental animals. In particular, recent findings regarding the detection of JCV genome in a variety of human tumors indicate that JCV may be associated with the induction of human tumors.

JCV is a small human DNA virus with a double-stranded, covalently linked circular genome, and is 5130 base pair in size. It is classified in the Papovaviridae family within the polyomavirus genus (Frisque, Bream, and Cannella, 1984). The JCV genome is composed of bidirectional regulatory elements and coding regions (Figure 1.2). The regulatory region contains the origin of viral replication and promoter elements for the transcription of viral regulatory and structural genes. The coding region can be divided into early and late coding regions. The early coding region primarily encodes the viral regulatory proteins, Large T and small t antigens, and T' proteins (T'165, T'136 and T'135) expressed by alternative splicing of the JCV-early primary transcript (Trowbridge and Frisque, 1995). The late coding region encodes a small regulatory protein, agnoprotein, and viral structural proteins (VP-1, VP-2 and VP-3), expressed by alternative splicing of the JCV-late primary transcript (Figure 1.2). A successful viral infection is strongly dependent on transcriptional activation of viral early and late promoters as well as on the splicing of primary early and late transcripts for the expression of viral structural and regulatory proteins. Several studies have revealed transcription factors which bind and

regulate JCV transcription, including NF-1, GF-1, Sp1, c-jun, YB-1 and Pura (Khalili and White, 2006). However, regulation of JCV gene expression by alternative splicing and the impact of alternative splicing factors on JCV propagation have not been documented.



**Figure 1.2: Schematic representation of the genomic organization of the JCV regulatory region and alternative splicing of JCV-early and -late transcripts.** JCV genome is composed of regulatory and coding regions. The regulatory region contains the origin of DNA replication and promoter/enhancer elements. The coding regions are divided into an early and late region. The early region encodes the regulatory proteins, large T antigen, small t antigen, and T' proteins. The late coding region encodes viral structural proteins (VP-1, VP-2 and VP-3) and a short regulatory peptide, agnoprotein. Splicing patterns of the individual proteins are schematized. Green color indicates the coding sequences

of the proteins. Red color indicates the 5', and 3'-UTRs. (Reference sequence: NC 001699). \

## **1.2 ASSOCIATION OF JCV WITH HUMAN TUMORS.**

### **1.2.1 Tumor induction by JCV in experimental animals.**

Following its isolation, JCV has not only been shown to cause a variety of tumors in experimental animals (Walker et al, 1973; Varakis et al, 1978; London et al, 1978, 1983; Krynska et al, 1999) but has also been shown to have the ability to induce neoplastic cell transformation in tissue culture. Since JCV-induced tumors arise in tissues of neural origin (Walker et al, 1973; Varakis et al, 1978), tissue-specific expression of JCV regulatory region is thought to play a major role in this process. Inoculation of JCV into several experimental animal models, including hamsters, nonhuman primates, and transgenic mice, results in a variety of tumors depending on the animal type, age, and site of inoculation. For instance, more than 80% of newborn Syrian hamsters, when inoculated intracerebrally and subcutaneously with the Mad-1 strain of JCV, developed glioblastomas, neuroblastomas, and medullablastomas (Walker et al, 1973; Varakis et al, 1978). JCV was also inoculated intraocularly into newborn hamsters and resulted in abdominal neuroblastomas developing in several locations of the body (Walker et al, 1973).

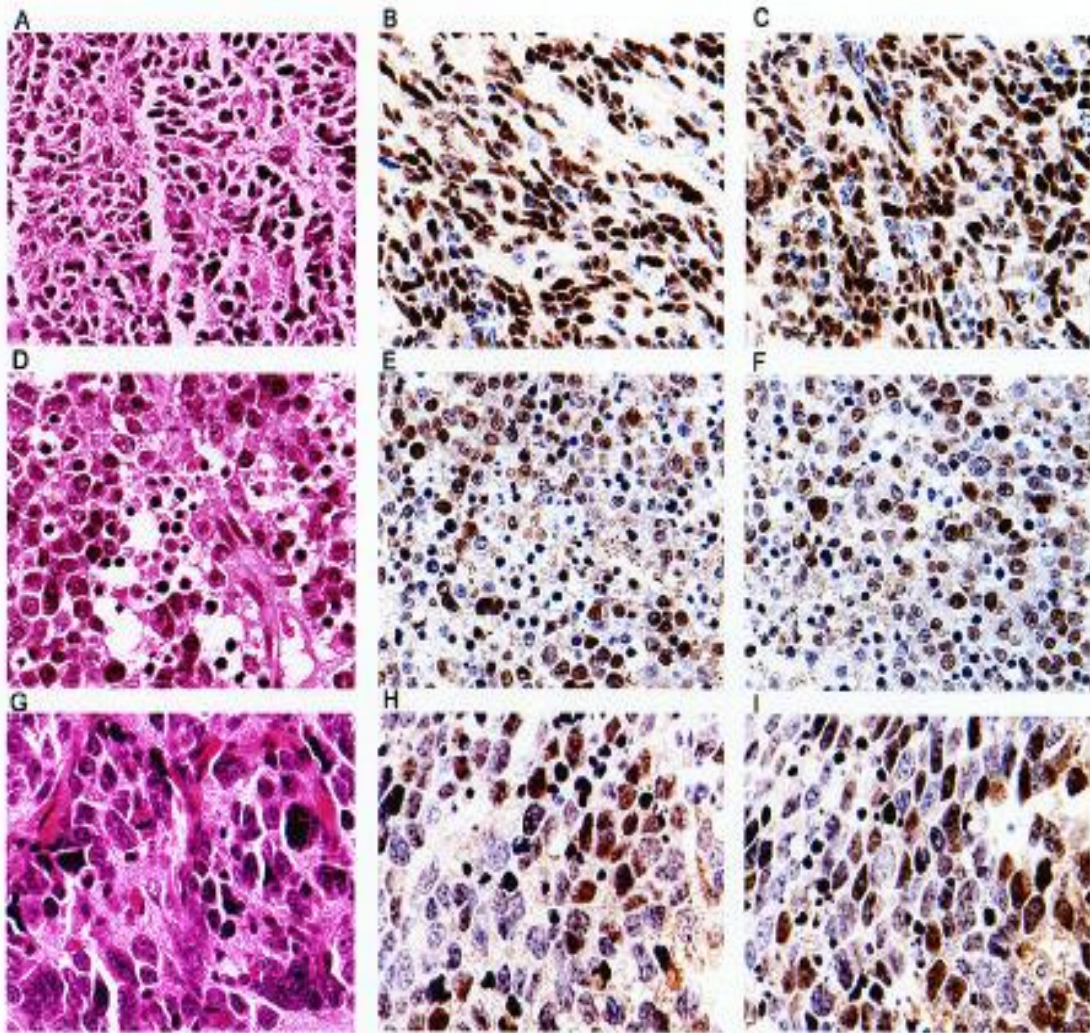
Unlike the other members of the polyomavirus family (BKV and SV40), JCV is the only polyomavirus shown to induce tumors in nonhuman primates, such as monkeys. When owl and squirrel monkeys were inoculated with live JCV

subcutaneously, intraperitoneally, and intracerebrally (London et al, 1978, 1983), the animals developed tumors at different time intervals. One owl monkey developed a malignant cerebral tumor similar to an astrocytoma seen in humans after 16 months of inoculation. Another one developed a malignant neuroblastoma 25 months after inoculation. Further analysis of tumors for the expression of JCV large tumor antigen (the main viral regulatory protein involved in tumor induction) revealed both the presence of large T antigen and complex formation with the tumor suppressor protein p53, (Dyson, 1990). Mechanistically, the tumorigenic potential of JCV T antigen appears to be, at least in part, mediated by its interaction with tumor suppresser genes, including p53 and retinoblastoma gene products, pRb and p130. Upon binding, T antigen appears to interfere with the cell cycle progression properties of these proteins. Large T antigen has also been shown to interact with cellular and viral proteins including YB-1, Pura, JCV agnoprotein, and insulin receptor substrate 1 (IRS-1) (Gallia, 1998; Safak et al, 1999, 2002; Lassak et al, 2002). IRS-1 is the major signaling molecule for the type I insulin-like growth factor receptor (IGF-IR) (Baserga, 1999).

In addition, recent reports also indicate a possible communication between JCV T antigen and the Wnt signaling pathway in induction of tumor formation because T antigen expressing cells express higher levels of  $\beta$ -catenin and its partner LEF-1 (Gan et al, 2001). Our group also described the formation of different tumors in tissues that derived from neural origin in transgenic mice models (Franks et al, 1996; Krynska et al, 1999; Gordon et al, 2000). The JCV

early coding region, driven by its own promoter, was utilized to create these transgenic animal models. In contrast to previous observations by Small et al (Small et al, 1986a,b), transgenic animals created with the early region of JCV archetype strain (Krynska et al, 1999) did not show any sign of hypomyelination in the central nervous system which was a feature observed in transgenic mouse models. On the contrary, cerebellar tumors that resemble human medulloblastomas appeared in the transgenic animals (Krynska et al, 1999).

In another line of transgenic mouse, half of the animals developed large, solid masses within the base of the skull by one year of age. Histological evaluation of the tumors by location and by histochemical studies demonstrated that these tumors arose from the pituitary gland (Gordon et al, 2000). Figure 1.4 exemplifies a variety of tumors induced by JCV in an experimental animal model system.



**Figure 1.3: JCV transgenic animal models.** Transgenic mice containing the full sequence of the JCV genome (archetype) develop primitive neuroectodermal tumors in the brain, characterized by numerous packed cells with elongated nuclei and scant cytoplasm (Panel A, Hematoxylin & Eosin). Immunohistochemistry against the early gene product T-antigen, demonstrates the nuclear localization of the protein (Panel B), and in the same group of cells, there is intense immunoreactivity for p53 (Panel C). Transgenic animals containing only the early sequence of JCV develop a variety of neural-origin tumors, including adrenal neuroblastomas, characterized by rounded

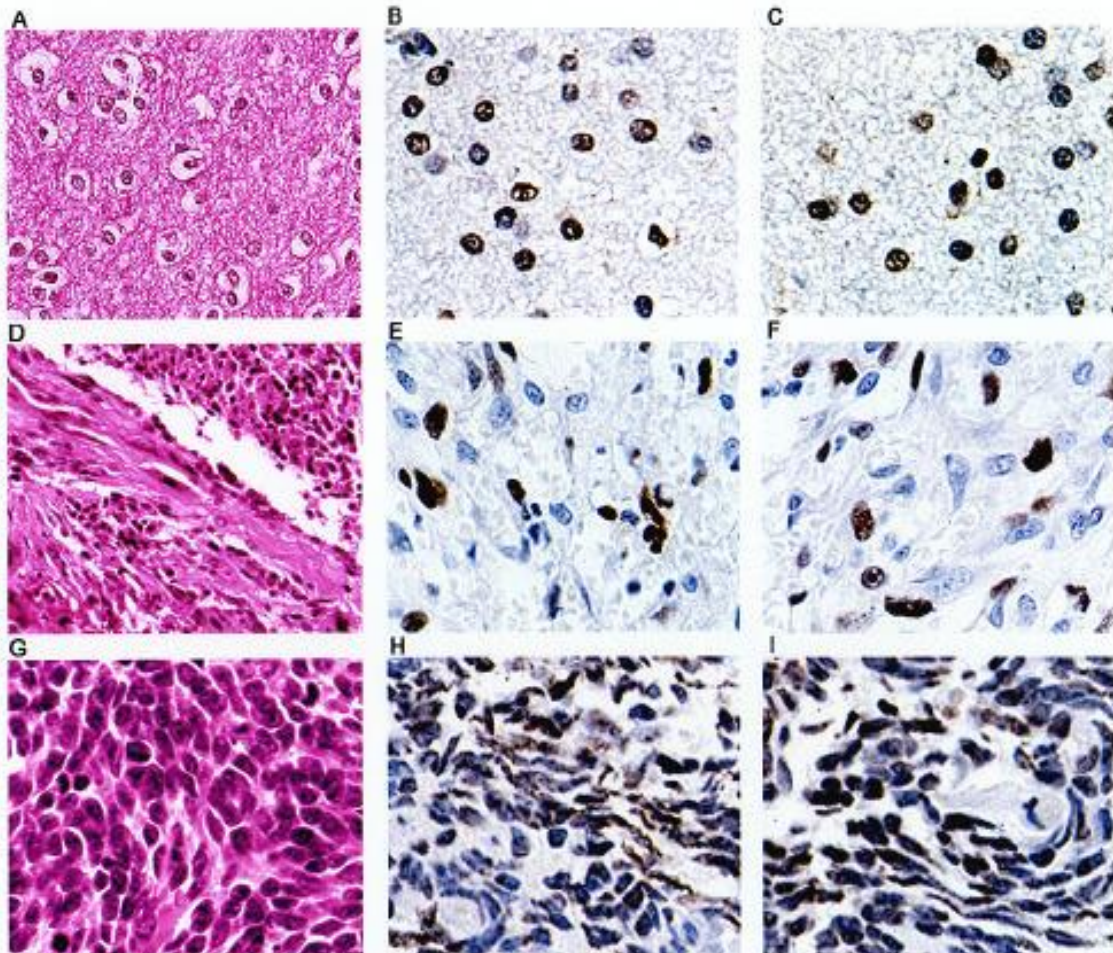
homogeneous cells with a perinuclear halo of cytoplasm (Panel D, Hematoxilin & Eosin), which also express nuclear Large T antigen when tested by immunohistochemistry (Panel E). In the same cellular compartment there is strong immunoreactivity for p53 (Panel F). Another tumor developed in a line of JCV early transgenic mice is pituitary adenomas, characterized by numerous pleiomorphic cells of different sizes and abundant eosinophilic cytoplasm (Panel G, Hematoxilin & Eosin). The neoplastic cells demonstrate intense nuclear positivity for T-antigen (Panel H), as well as p53 (Panel I). All panels are original magnification x1000 (Sariyer et al., 2004).

### **1.2.2 Detection of JCV in human tumors.**

In recent years, a widespread detection of the JCV genome in variety of human tumors raised the possibility that JCV may induce tumors in humans. In fact, Richardson, who first described PML in 1961 (Richardson, 1961), reported the incidental detection of an oligodendroglioma in a patient with concomitant occurrences of chronic lymphatic leukemia and PML. Following this report, concomitant occurrences of PML with different human tumors was described in several more cases. Sima et al., reported the association of PML with multiple astrocytomas in 1983 (Sima, 1983). Similarly, Casteigne et al., (1974) described a case where a patient with a long history of immunodeficiency syndrome, in addition to PML, showed numerous foci of anaplastic astrocytes. Microscopic analysis of the demyelinating lesions demonstrated the presence of viral particles in both oligodendrocytes and astrocytes within PML foci, but not in the neoplastic

astrocytes (Casteigne, 1974). A more recent report by Shintaku and colleagues showed dysplastic ganglion-like cells in a patient with PML (Shintaku et al, 2000). A large number of dysplastic or dysmorphic ganglion-like cells were found in the cerebral cortex that showed properties of neurons. Expression of the JCV large T antigen was demonstrated in the infected neurons, however, the late gene products were not. In addition to the cases described above, JCV genome has also been detected in human brain tumors in the absence of PML lesions. Boldorini et al., reported the detection of JCV DNA in the brain tumors of an immunocompetent patient with a pleiomorphic xanthoastrocytoma (Boldorini et al, 1998). An earlier study by Rencic et al, demonstrated the presence of JCV viral DNA and expression of large T antigen in tumor tissue from an immunocompetent HIV-negative patient with oligoastrocytoma (Rencic et al, 1996). These two cases presented the experimental evidence for a possible association of JCV in brain tumors of immunocompetent non-PML patients. Such findings further prompted the attempts to establish the association of JCV with different types of brain tumors in humans. In fact, Del Valle et al, (Del Valle et al., 2002; Del Valle et al., 2001) recently analyzed multiple brain tumors for the detection of the JCV genome and showed that 62.5% of oligoastrocytomas, 83.3% of ependymomas, 80% of pilocytic astrocytomas, 57.1% of oligodendrogliomas, 76.9% of astrocytomas, and 66% of anaplastic oligodendrogliomas contained JCV early gene sequence. Figure 1.5 illustrates the detection of the JCV early oncogenic protein, large T antigen, and cellular tumor suppressor protein, p53, in a variety of human tumors. JCV genomic DNA

has also been shown to be present in tumor tissue which is not of neural origin. Recent reports indicate that the JCV genome was detected in the gastrointestinal tract and solid non-neural tumors, including colorectal cancers (Laghi et al., 1999; Ricciardiello et al., 2000, 2001; Enam et al., 2002). It should be however noted here that such studies explored the possibility of whether JCV genome or its expressed proteins could be detected by certain molecular biology techniques but does not provide information about the mechanism by which JCV could possibly induce tumors in humans.



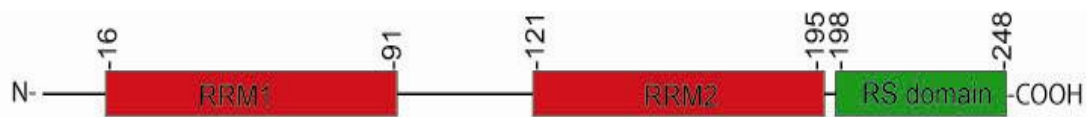
**Figure 1.4. Detection of JCV proteins in human brain tumors.** Expression of JCV early proteins has been found in a wide variety of brain neoplasms,

including low grade glial tumors, such as oligodendrogliomas (Panel A, Hematoxylin & Eosin), characterized by homogeneous cells with a clear halo surrounding their nuclei. Immunohistochemistry for large T-antigen is positive in the nuclei of the majority of the neoplastic cells (Panel B), where the cell cycle regulator protein p53 is also found (Panel C). High-grade glial tumors such as glioblastoma (Panel D) characterized by extensive areas of necrosis and pleomorphic, atypical cells expressing T antigen in their nuclei (Panel E). p53 is also present in the nuclei of the neoplastic cells (Panel F ). Tumors of neural origin, such as medulloblastomas, characterized by numerous sheaths of homogeneous cells, with scant cytoplasm (Panel G, Hematoxylin & Eosin), demonstrate nuclear expression of the early JCV protein, T-antigen (Panel H), and also nuclear immunoreactivity for p53 (Panel I). All panels are original magnification x1000 (Sariyer at al., 2004).

### **1.3 SF2/ASF**

Alternative splicing of primary transcripts is a widely used mechanism to increase the coding capacity of the genes in mammalian cells. Several protein factors play important roles in the complex regulation of pre-mRNA splicing. Among these factors, the Ser/Arg-rich proteins (SR proteins) are phylogenetically conserved proteins originally identified for their regulatory roles in alternative and constitutive splicing of mRNA precursors (Licatalosi and Darnell, 2010). SF2/ASF (splicing factor 2/ alternative splicing factor) is a prototypic member of this family and is one of the key regulators of alternative splicing (Manley and Tacke, 1996).

SF2/ASF has a modular structure consisting of two copies of N-terminal RNA-recognition motifs (RRM1, RRM2), followed by a C-terminal domain rich in Arg and Ser residues known as the Arginine-Serine-rich (RS) domain. The RS domain interacts with the components of the core splicing apparatus to form splice site pairing, whereas the RRM1 and RRM2 domains determine the RNA-binding specificity ( Figure 1.5) (Graveley et al., 2000; Sanford et al., 2005).



**Figure 1.5: Modular structure of SF2/ASF.** RRM1: RNA recognition motif 1, RRM2: RNA recognition motif 2, RS: Arginine/serine rich domain.

SF2/ASF is mainly localized to nuclear speckles and shuttles between the cytoplasm and nucleus (Caceres et al., 1997). Depending on splice site selection, SF2/ASF can negatively or positively regulate splicing (Zuo and Manley, 1993; Wang and Manley, 1995). Beside the roles of SF2/ASF in the regulation of gene expression through the modulation of pre-mRNA alternative splicing, it has also been shown to be an inducer of translation initiation by suppressing the activity of 4E-BP1, an inhibitor of cap-dependent translation (Michlewski et al., 2008). SF2/ASF has also been shown to be an essential host protein in light of data demonstrating that SF2-deficient mice show embryonic lethality (Xu et al., 2005). In addition to its role in gene expression through the

regulation of splicing and translation, SF2/ASF is also up-regulated in various human tumors, including lung, kidney, colon, pancreas, small intestine, and thyroid. Up-regulation of SF2/ASF in tumor cells indicates a possible role in malignant transformation. Furthermore, over-expression of SF2/ASF is sufficient to transform NIH- 3T3 and Rat1 fibroblasts, and its transforming activity is related to aberrant splicing of the tumor suppressor, BIN1, and the kinases, MNK2, and S6K1, (Karni et al., 2007).

Not surprisingly, SF2/ASF is utilized by many viruses (i.e., adenovirus, human papillomavirus, Rous sarcoma virus, influenza virus, herpes simplex virus, and HIV-1) in order to regulate their gene expression upon infection of host cells. It has been shown that SF2/ASF binds the human papillomavirus type 16 late RNA control element, HPV-16 NRE and its expression is up-regulated by viral E2 protein during the differentiation of epithelial cells infected with the virus (Mole et al., 2009; McPhillips et al., 2004). Gene expression of HIV-1 is also influenced by SF2/ASF. The 9kb full-length HIV genomic transcript undergoes a series of posttranscriptional modifications that generates viral mRNAs that produce viral structural and regulatory proteins. SF2/ASF binds to exonic splicing enhancers (ESEs) downstream of the Tat-, Rev-, Env splice sites and promotes exon definition (Bakkour et al., 2007; Caputi et al., 2004; Powell et al., 1997; Luo et al., 1994; Keriell et al., 2009). Therefore, SF2/ASF is thought to play a major role in the regulation of HIV-1 pre-mRNA splicing and has been proposed as a novel target for the inhibition of HIV replication (Bakkour et al., 2007).

SF2/ASF was first discovered as a cell-type specific regulator of SV40-early region gene expression at the post-transcriptional level (Ge et al., 1990). Early studies using in vitro transcribed SV40-early primary transcript and cellular extracts have suggested that SF2/ASF enhances the usage of small t antigen splice site at the expense of the Large T antigen site (Manley and Ge, 1990). However, in vivo studies have revealed that over-expression of SF2/ASF inhibited not only Large T but also small t, and caused accumulation of unspliced RNA (Manley and Wang, 1995). These studies suggest that alternative splicing of viral-early and -late primary transcripts and their regulation by alternative splicing factors, particularly by SF2/ASF, might be a critical determinant of successful viral replication.

#### **1.4 HYPHOTHESIS AND PROPOSED AIMS OF THE STUDY**

The main limitations of JCV studies are the absence of an animal model system and a reliable cell line which supports JCV replication. Currently, POJ and SVG-A cell lines are used for JCV propagation or for the study of its biology. These cell lines were generated from the primary fetal glial cells by transformation process using either origin defective JCV (POJ) (Mandl, Walker, and Frisque, 1987) or SV40 (SVG-A) (Major et al., 1985). Although both lines efficiently support virus growth, their application for the study of the molecular mechanisms involved in the viral infection cycle is limited due to the constitutive expression of large T antigen. Beside transformed cell lines, primary human fetal glial cells can be used to study the life cycle of JCV; however, due to high cost

and labor intensity associated with the preparation of these cultures, researchers are looking to develop new alternative culture systems for understanding JCV biology.

Cell fusion is a normal developmental process that is important for diverse functions, including fertilization, skeletal muscle development, bone formation, and the immune system (Larsson, Bjerregaard, and Talts, 2008). In addition to normal fusion events during development, some viruses (fusogenic viruses) are capable of causing cell-to-cell fusion and this may contribute to cell transformation due to genomic instability (Duelli et al., 2005; Duelli et al., 2007; Hernandez et al., 1996). In practice, cell-to-cell fusion has been achieved between somatic and cancer cells in order to obtain immortal somatic hybrid clones (Dunnion et al., 1999; Jantscheff et al., 2002; Shirahata, Katakura, and Teruya, 1998; Stefano et al., 1993). A well-known example of an in vitro cell fusion process is the creation of hybrid clones between myeloma and spleen cells for the production of monoclonal antibodies (Kohler and Milstein, 1975). The new lines gain immortality and express antibody from a B-cell lineage. Hybrid clones created using somatic and cancer cells typically gain the properties of both parental cells, such as expressing specific genes from both lines (Nagai et al., 2002; Raje et al., 2004; van Olphen and Mittal, 2002).

I propose to create hybrid cell lines using JCV-permissive primary human fetal astrocytes (PHFA) and a human malignant glioma cell line, U-87MG. First, we will create HPRTase deficient U87 MG subclones by 8-azoguanine selection. Then, we will use this cell line to fuse with PHFA cells by PEG-mediated cell

fusion. Hybrid cell lines will be obtained by limiting dilution, \ selected by JCV infection, and characterized.

The human polyomavirus, JC virus (JCV), is the etiological agent of progressive multifocal leukoencephalopathy (PML) characterized by the lytic infection of oligodendrocytes, the myelin-producing cells in the central nervous system. JCV is presumed to remain latent, and it reactivates under immunosuppressive conditions. Productive infection of JCV occurs only in glial cells and this restriction is, to a great extent, due to the activation of the viral promoter that has cell type-specific characteristics. Earlier studies led to the assumption that glial-specific activation of the JCV promoter is mediated through the involvement of positive and negative transcription factors that control reactivation of the JCV genome under normal physiological conditions and suppress its activation in non-glial cells.

SF2/ASF was first discovered as a cell-type specific regulator of SV40-early region gene expression at the post-transcriptional level (Ge et al., 1990). Early studies using in vitro transcribed SV40-early primary transcript and cellular extracts have suggested that SF2/ASF enhances the usage of small t antigen splice site at the expense of the Large T antigen site (Manley and Ge, 1990). However, in vivo studies have revealed that over-expression of SF2/ASF inhibited not only Large T but also small t and caused accumulation of un-spliced RNA (Manley and Wang, 1995). These studies suggest that alternative splicing of viral-early and -late primary transcripts, and their regulation by alternative

splicing factors, particularly by SF2/ASF, might be a critical determinant of successful viral replication.

First, I propose to investigate the impact of SF2/ASF on JCV replication and gene expression in glial cells. These experiments will be performed in primary human fetal glial cells, PHFG and SVGA cells, both of which are permissive for JCV infection. The roles of SF2/ASF on JCV replication and transcription will be analyzed during the life cycle of the virus by perturbing its expression level using expression plasmids or siRNAs.

Second, I propose to investigate the molecular mechanisms of SF2/ASF in the negative regulation of JCV. We will investigate the effect of SF2/ASF on transcriptional activity of the JCV-early and –late promoter in the presence or absence of the viral regulatory protein, LT-ag. Chloramphenicol acetyl transferase assays (CAT) will be performed to analyze the influence of SF2/ASF on viral promoter activities. We will also create truncated forms of SF2/ASF to determine the functional domain within the protein that suppresses viral transcriptional activity. We will also analyze the direct or indirect interaction between SF2/ASF and the JCV promoter. We will perform three sets of DNA binding experiments. In the first set of experiments, we will employ DNA-footprinting techniques using the 373bp DNA probe spanning the entire JCV promoter. The second approach includes the band shift assay, which uses the oligoes, covering the protected JCV promoter regions from footprinting studies. Finally in the third series of DNA binding studies, we will utilize chromatin

immunoprecipitation (ChIP) assays that allow in vivo analyses of DNA-protein complexes.

Third, I propose to investigate the hypothesis that SF2/ASF inhibits JCV early gene expression in tumor cell lines obtained from transgenic animals. These experiments will be performed in BsB8 and HJC2 cells which express the JCV early genes, Large T antigen and small t antigen, under the JCV promoter. We will analyze the down-regulation of JCV early gene expression by SF2/ASF in BsB8 and HJC-2 cells using expression plasmids. We will investigate the role of SF2/ASF-mediated JCV early gene extinction on the transformed phenotype of the BsB8 and HJC-2 cells. Colony formation assays and growth in soft agar will be employed to explore the effect of SF2/ASF on the transformed phenotype of these cell lines.

Through these aims, we will create an in vitro hybrid-cell line system which supports JCV replication, and will test the hypothesis that SF2/ASF is one of the major cellular players in the control of JCV gene expression. This will provide important information on how JCV gene expression is regulated in glial cells in the context of PML development and how viral tumor proteins maintain the transformed phenotype in JCV-induced tumors.

**CHAPTER 2**  
**MATERIALS AND METHODS**

**2.1 Plasmid Constructs.**

pCGT7-SF2/ASF-FL expression plasmid was kindly provided by Javier F. Ca´ceres (Medical Research Council Human Genetics Unit, Western General Hospital, Edinburgh EH4 2XU, Scotland, United Kingdom) and was described previously (Cazalla et al., 2002). All of the new plasmid constructs and primer pairs created and used in this dissertation are listed in table 2.1.

Table 2.1: Plasmid constructs and primers.

PCGT7-SF2/ASF Antisense	Forward	ACCTTCCAGGATCCATGTCGGGAGGTGGTGTGATT
	Reverse	ACCTTCCATCTAGATTATGTACGAGAGCGAGATCT
pCGT7-SF2/ASF Mut.RS	Forward	ACCTTCCAGGATCCATGTCGGGAGGTGGTGTGATT
	Reverse	ACCTTCCAGGATCCTTACCCATCAACTTTAACCCG
pCGT7-SF2/ASF RRM1	Forward	ACCTTCCAGGATCCATGTCGGGAGGTGGTGTGATT
	Reverse	ACCTTCCAGGATCCTTAGCCGCCGCTCGGCCTGT
pCGT7-SF2/ASF Mut.RRM1	Forward	ACCTTCCATCTAGAGCGGGGGTGGAGGTGGCGGA
	Reverse	ACCTTCCATCTAGATTATGTACGAGAGCGAGATCT
pCGT7-SF2/ASF RS	Forward	ACCTTCCATCTAGACCCAGAAGTCCAAGTTATGGA
	Reverse	ACCTTCCATCTAGATTATGTACGAGAGCGAGATCT
pBLCAT3-Mad1 RR (4987-248)	Forward	ACCTTCCAGGATCCTTCCTCCCTATTCAGCAC TT
	Reverse	ACCTTCCACTCGAGTCTGGCTCGCAAACATGTTC

Blue-Mad1 (1X98)	Forward	GGAAGTGGAAAGCAGCCAAGGGAACATGTT
	Reverse	CCTTGTGCTTTGTTTACTGGCTGTTAGCTGG
Blue-Mad1 (1x98)Mut.CR3	Forward	ACAGCCAGTAAACAAAGCACAAGGGGAAGTGGA
	Reverse	GCTCATGCTTGGCTGGCAGCCATCCCTTCCCTT
pBLCAT3-pSF2 (-1500 to +47)	Forward	ACCTTCCAAAGCTTGGTATCTCTGTAAGTTGCCTC
	Reverse	ACCTTCCACTCGAGGAAGGAAACAGCGATTTCGATC
pBLCAT3-pSF2 (-1000 to +47)	Forward	ACCTTCCAAAGCTTTCCAGATTTTCAGACGTCGAAA
	Reverse	ACCTTCCACTCGAGGAAGGAAACAGCGATTTCGATC
pBLCAT3-pSF2 (-500 to +47)	Forward	ACCTTCCAAAGCTTAATTAGACACATCTGTCTAAG
	Reverse	ACCTTCCACTCGAGGAAGGAAACAGCGATTTCGATC
pBLCAT3-pSF2 (-400 to +47)	Forward	ACCTCCCAAGG CTTTGGATTAGACGCACCCTACGA
	Reverse	ACCTTCCACTCGAGGAAGGAAACAGCGATTTCGATC
pBLCAT3-pSF2 (-300 to +47)	Forward	ACCTCCCAAGGCTTGTTATCGCCGCAGAGCATGGT
	Reverse	ACCTTCCACTCGAGGAAGGAAACAGCGATTTCGATC
pBLCAT3-pSF2 (-200 to +47)	Forward	ACCTCCCAAGGCTTATCGACCATCCTTCAAGACCC
	Reverse	ACCTTCCACTCGAGGAAGGAAACAGCGATTTCGATC
pBLCAT3-pSF2 (-100 to +47)	Forward	ACCTCCCAAGGCTTCCCAAGCTGGGAACGCGGGG
	Reverse	ACCTTCCACTCGAGGAAGGAAACAGCGATTTCGATC
pBLCAT3-pSF2 (-50 to +47)	Forward	ACCTCCCAAGGCTTGACGTCGCGCGTGCGTGCGCG
	Reverse	ACCTTCCACTCGAGGAAGGAAACAGCGATTTCGATC

## 2.2 Cell cultures.

The human malignant glioma cell line, U87-MG, was obtained from American Type Culture Collection (ATCC), and was grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% heat-inactivated fetal bovine

serum (FBS) and penicillin/streptomycin (100µg/ml). Primary human fetal glial cells were prepared as previously described (Radhakrishnan et al., 2003). Briefly, human fetal brain tissue was obtained from Advanced Biosciences Resources, Inc. (Alameda, CA). The tissue was washed with HBSS medium and placed in a 100 mm dish. Blood vessels and meninges were dissected, and tissue was cut into small pieces using a forceps and scalpel. Chopped-tissue was mechanically disrupted by pipetting up and down in HBSS with a 10 ml pipette until cell culture fluid smooth and pinkish in color. The tissue was centrifuged and digested with DNase I and trypsin in 10 ml HBSS medium for 30 minutes at 37 °C. Cells were washed with HBSS and passed through a 70-micron filter. Mixed cultures of glial cells were plated in Poly-D-Lysinized T162 cm<sup>2</sup> flasks with DMEM/F12 medium (1/1) containing 10% FBS, 1% L-glutamine, 1% Fungizone, insulin, and gentamycin. After plating 4-5 days, the cells were washed with PBS and trypsinized. They then were plated in T162 cm<sup>2</sup> flasks and incubated for 45 minutes. During the 45 minute period, microglial cells attached to the flask, and most of the astrocytes, neurons, and oligodendrocytes remained in the medium. After 45 minute incubation, the medium was removed and placed in new flasks. The cells were grown in culture until they were confluent. Once confluent, the cells were placed on an orbital shaker to remove the neurons and oligodendrocytes, which detached from the surface of the flasks and came off into the medium. After proper shaking, the medium was replaced with astrocyte growth medium, DMEM/F12 (1/1) with 15% FBS, 1% L-glutamin, insulin, and gentamycin. Primary human adult astrocytes were purchased from ACBRI and

maintained in DMEM/F12 (1/1) growth medium containing 10% FBS, 1% L-glutamin, 1% Fungizone, insulin, and gentamycin. BsB8 cells were derived from cerebellar medulloblastomas that developed in transgenic mice expressing the early genome of the JC virus (Krynska et al., 2000). HJC-2 (Wold et al., 1980) is a clonal subline of the HC-15 cell line which was established from a hamster brain tumor (a mixed malignant glioma of astrocytic and ependymal derivations) induced by intra-cerebral inoculation of neonatal hamsters with JCV (Walker et al., 1973). SVG-A is a human glial cell line which was established by transformation of primary human fetal glial cells with an origin-defective SV40 virus and described previously (Major et al., 1985). All of the cell lines and cultures were maintained at 37 °C in a humidified atmosphere with 7% CO<sub>2</sub>.

### **2.3 JCV infection.**

Transfection/infection of cells with the full-length JCV Mad-1 genome was described previously (Sariyer et al., 2006, and 2009). Briefly, PHFA cells, at a confluence of  $1 \times 10^6$  cells per T75-cm tissue culture flask, were co-transfected/infected with the full-length JCV DNA (10 µg/flask) in the presence or absence of pCGT7 plasmid expressing SF2/ASF in the sense or antisense orientation using Fugene 6 transfection reagent as indicated by the manufacturer (Roche). At different days post-infection, cells were trypsinized and split into two equal portions. One half was used for preparation of whole cell protein extract for Western blot analysis, and the other half was split into two equal volumes for DNA and RNA preparation using Qiaprep Spin Miniprep kit (Qiagen), and

RNeasy Mini kit (Qiagen), respectively. Primary astrocytes, HC-7 and HC-15 hybrid clones ( $1 \times 10^6$  cell/75-cm<sup>2</sup> flask) were infected with the Mad-1 strain of JC Virus (30 HAU/cell) and fed with DMEM supplemented with 2% FBS and antibiotics (penicillin-streptomycin, 100 µg/ml). Cells were then incubated at 37°C in a humidified atmosphere with 7% CO<sub>2</sub> until they were needed for extract preparation or DNA isolation.

#### **2.4 DpnI assay and detection of replicated-viral DNA by Southern blotting.**

Low molecular weight DNA purified from JCV-infected cells by transfection/infection method was digested with Dpn I and BamH1 enzymes. Digested DNA samples were separated on 1% agarose gel and were transferred to a nylon membrane. Replicated viral DNA was visualized upon incubation of the membrane with a [<sup>32</sup>P]-labeled JCV DNA probe as described earlier (Sariyer et al., 2008, and 2009).

#### **2.5 RT-PCR.**

Total cellular RNA was extracted from JCV-infected PHFA cells by using the Qiagen RNeasy kit according to the manufacturer's recommendations. After treatment with DNase I, followed by phenol/chloroform extraction and ethanol precipitation, cDNAs were synthesized using M-MuLV reverse transcriptase. RNA templates were removed by RNase H digestion. A total of 100 ng cDNA was used as a template for PCR reactions. Viral transcripts from JCV-infected

cells were determined by using primers listed in Table 2.2. To analyze the effect of SF2/ASF on JCV early gene expression, PHFA and BsB8 cells were transfected with pCGT7-control or with pCGT7-SF2/ASF expression plasmids. At 48 h post-transfection, cells were harvested, and total RNA was extracted with an RNA extraction kit (RNeasy, Qiagen) according to the manufacturer's instructions. One microgram of RNA was then reverse-transcribed using M-MuLV-reverse transcriptase and oligo-dT primers. JCV Mad-1 genomic DNA and U87-MG cells were used as positive and negative controls of amplifications, respectively. RT-PCR reactions of the JCV-early region splicing were performed by JCV-early PF and PR primer pair (table 2.2).

**Table 2.2: Primer sequences used in RT-PCR and Q-PCR reactions.**

JCV-VP1	Forward	CTCATGTGGGAGGCTGTGAC
	Reverse	TCCTCCTGTTAGTGTC CCAA
JCV-Large T antigen	Forward	ATGGACAAAGTGCTGAATAGG
	Reverse	TAGTGGTATACACAGCAAAAG
GAPDH	Forward	TTCTCCCATTCCGTCTTCC
	Reverse	GTACATGGTATTCACC ACCC
JCV-early PF (4801-4780)	Forward	CCTGATTTTGGTACATGGAA
JCV-early PR (4291-4313)	Reverse	GTGGG GTAGAGTGTTGGGATCCT
JCV-VP1 Q-PCR	Forward	AGTTGATGGGCAGCCTATGTA
	Reverse	TCATGTCTGGGTCCCCTGGA

## **2.6 Detection of viral particles in culture medium by Q-PCR.**

Transfection/infection of cells with the full-length JCV-Mad1 genome was performed as described above. The culture medium (containing viral particles) was collected at 8 days post-infection, and after centrifugation at 10,000 rpm for 10 minutes to remove cell debris, supernatants were collected and incubated at 95°C for 10 minutes to inactivate virus. Ten microliters of the medium was then used as a template in Q-PCR reactions. The standard curve was obtained after serial dilution of pJCV, a plasmid containing the whole genome of the JCV Mad-1 strain, knowing that 10 ng of pJCV correspond to 10<sup>9</sup> copies of viral genome. The standard curve was then used to extrapolate the viral load of each sample. Negative and positive controls were included in each reaction and each sample was tested in triplicate. All Q-PCR analyses were done by using Lightcycler 480 (Roche). Primers were JCV Q-PCR-forward and JCV Q-PCR-reverse (table 2.2). The probe for the Q-PCR was 5'-/5HEX/CATGGATGCTCAAGTAGAGG AGGTTAGAG TTT/3BHQ\_1/-3'.

## **2.7 Reporter gene assays.**

Reporter gene constructs containing the regulatory region of the JCV Mad-1 strain was described previously (Akan et al., 2006). Briefly, the Mad-1 (4989 to 480) region was PCR-amplified and inserted into the BamHI site of the pBLCAT<sub>3</sub> vector in early and late orientations. The resulting plasmids were called pBLCAT<sub>3</sub>-Mad1-Early and pBLCAT<sub>3</sub>-Mad1-Late. pBLCAT<sub>3</sub>-BKV-Early and pBLCAT<sub>3</sub>-BKV-Late plasmids were described previously (Luckow and Schutz,

1987). PHFA cells were transfected with these constructs in the presence or absence of expression plasmids for SF2/ASF-FL and its mutant forms. At 48h post-transfection, cells were extracted with a series of freeze/thaw cycles, and the CAT activity of the samples was determined.

## **2.8 Immunocytochemistry.**

In order to show localization of SF2/ASF in glial cells, PHFA cells were seeded in two-well chamber slides and transfected with pCGT7-SF2-FL, pCGT7-SF2-Mut.RS, pCGT7-SF2-Mut.RRM1, and pCGT7-SF2-RRM1 plasmids. After 48h, cells were fixed with cold acetone/methanol (1/1) for 2 minutes and washed three times with PBS. Cells were treated with a 5% BSA solution, followed by incubation with T7 monoclonal antibody. Cells were then incubated with FITC-conjugated secondary antibody, mounted with aqueous mounting medium, and examined under fluorescence microscope. To analyze infection of PHAA and PHFA with JCV, cells were plated in 2-well chamber slides at 10 days post-infection and fixed with cold acetone/methanol (1/1). After treatment with a 5% BSA solution, samples were incubated with JCV VP1-specific monoclonal antibody followed by incubation with FITC-conjugated secondary antibody. Samples were mounted with aqueous mounting with DAPI, and examined for microscopic immunofluorescence. In order to analyze the localization of JCV proteins in newly generated hybrid cells, PHFA and hybrid clones (HC-7, HC-15) were infected with Mad1 strain of JC Virus. Infected cells were then seeded on two-well chamber slides. After 24 hours incubation, cells were washed with PBS

and fixed with cold acetone at 8 days of infection. Fixed cells were incubated with 8% bovine serum albumin in PBS for 3 h and incubated with anti-Large T monoclonal antibody (Ab2), anti-VP1 (Pab597) and anti-agnoprotein polyclonal antibodies ( Pab7902) for 4 h. Cells were washed with PBS and incubated with FITC-conjugated secondary antibodies for 1 h. Finally cells were mounted and examined by indirect immunofluorescence microscopy.

## **2.9 Production and purification of GST proteins.**

SF2/ASF was amplified with the following primers, GST-SF2 primers (table 2.1), and cloned into BamHI and EcoRI sites of the pGEX2T E-coli expression vector. Bacterial cultures were incubated in one liter LB growth medium at 37 °C until they reached a confluence of 0.6 at 595.0 nm wavelength (wl) after which they were transferred to 28 °C and treated with IPTG (5 µg/ml). Cultures were centrifuged at 5k rpm for 10 min and re-suspended in 20 ml of PENT buffer (20 mM tris-HCL pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5 % NP-40 1 % N-L-sarcosyl, and general protease inhibitors), after which they were lysed by sonication. Bacterial lysates were centrifuged at 15k rpm for 15 minutes. Clear lysates were incubated with 150 µl of glutathione sepharose beads overnight, centrifuged at 3k rpm for 5 minutes, and resuspended in TNN buffer (150 mM NaCl, 40 mM Tris pH 7.4 , 1% NP-40 ( ipegal ), 1 mM DTT, 1 mM EDTA). Beads were washed in TNN buffer three times, and resuspended in PBS. The integrity of the proteins was tested by SDS-PAGE. SF2/ASF-FL protein was cleaved from the GST fusion protein by thrombin, and was subsequently used in gel shift experiments.

## **2.10 Chromatin immunoprecipitation, (ChIP) assay.**

BsB8 cells were transiently transfected with pCGT7-SF2/ASF full-length and its truncated forms. ChIP assays were performed as described previously (Kinoshita and Jhonson, 2004). Briefly, proteins were cross-linked to DNA by formaldehyde, followed by sonication to fragment the chromatin and immunoprecipitation of specific proteins to obtain DNA segments. Cross-linking was reversed and DNA was analyzed by PCR.

## **2.11 RNA interference.**

To knock-down expression of SF2/ASF, PHFA cells were seeded ( $5 \times 10^5$  cells per well) in 60 mm dishes. After 24 h, cells were transfected with 200 pmol short interfering RNA (siRNA) per well (Santa Cruz), using oligofectamine (Invitrogen). After 72 h, total proteins were prepared. Lentivirus-based U6-promoted SF2 shRNA constructs were generated by cloning PCR products carrying sense and antisense oligonucleotides of SF2/ASF into the pLL3.7 vector as described previously (Song et al., 2007; Rubinson et al., 2003). Two SF2 shRNA constructs were made to target the nucleotide sequences 35–55 (shA), and 423-443 (shB) of the human SF2 cDNA (GenBank accession number AAH33785.1). The viruses were packaged in 293T (human embryonic kidney) cells according to the procedure described previously (Rubinson et al., 2003). Primary human fetal and adult astrocytes were plated in six-well plates at 50% confluence and were incubated with 1ml of viral supernatants. The infected cells were then kept in regular complete medium for 48 h. Inspection with fluorescence

microscopy confirmed the presence of more than 80% of GFP-positive cells after viral infection. The level of SF2 in infected cells was evaluated by Western blot analysis.

## **2.12 Gel shift assay.**

The oligonucleotides used in band shift assays are listed in table 2.3. Band shift assays were carried out as described previously (Sariyer et al., 2008; Romagnoli et al., 2008). Recombinant SF2 or nuclear extracts from PHFA were incubated with [<sup>32</sup>P]-end labeled oligonucleotides (50,000 cpm/reaction) in a binding buffer containing 1.0 µg poly (dI-dC), 12 mM HEPES (pH 7.9), 4 mM Trish [pH 7.5], 60 mM KCl, 5 mM MgCl<sub>2</sub> and 1.0 mM DTT. The reaction mixture was incubated at 4 °C for 1h to allow assembly of DNA-protein complexes. The complexes were then resolved on a 6% polyacrylamide gel in 0.5× TBE (1× TBE is 89 mM Tris- HCl [pH 8.0], 89 mM boric acid and 2 mM EDTA [pH 8.0]). The gels were dried, and complexes were detected by autoradiography. The presence of SF2/ASF in the CR3/nucleoprotein (CR3/NP) was analyzed by gel shift followed by Western blot analysis. End-labeled or unlabeled CR3 oligonucleotide was incubated with nuclear extracts from PHFA cells and complexes were resolved on 6% native PAGE. End-labeled CR3/NP complexes were used as reference for unlabeled-CR3/NP complexes, and the DNA/protein complexes were cut from the gels, and elution of its content was analyzed by SDS-PAGE followed by Western blot analysis using anti-SF2/ASF antibody.

**Table 2.3: Oligonucleotide sequences used in gel shift experiments.**

Kb-JCV-Mad1 (5052-5078)	AAAACAAGGGAATTTCCCTGGCCTC
ORI-JCV-Mad1 (5118-12)	GGAGGCGGAGGCGGCCTCGGCCTCC
CR1-JCV-Mad1 (11-35)	CCTGTATATATAAAAAAAGGGAAG
CR2-JCV-Mad1 (36-60)	GGATAGCTGCCAGCCAACGATGAGC
CR3-JCV-Mad1 (61-85)	TCATACCTAGGGAGCCAACCAGCTA
CR4-JCV-Mad1 (86-110)	ACAGCCAGTAAACAAAGCACAAGGC

### **2.13 Colony formation assay.**

BsB8 and HCJ-2 cells ( $5 \times 10^5$  / 100mm dishes) were co-transfected either with pCGT7 empty vector or pCGT7-SF2/ASF and pcDNA 3.1 zeo(+) plasmids. After 24h, transfected and untransfected control cells were collected by trypsinization and re-plated in 100 mm dishes ( $5 \times 10^4$  / 100mm dishes) in DMEM medium containing 10% FBS and G418 (1mM). Cells were maintained for three weeks, and the number of colonies was determined by staining the cells with 1% methylene blue. Experiments were performed in triplicate.

### **2.14 Soft agar growth assay.**

BsB8 and HCJ-2 cells ( $3 \times 10^5$  / 60mm dishes) were co-transfected with pCGT7-SF2/ASF and pcDNA 3.1 zeo (+) plasmids. After 24h, transfected and untransfected control cells were harvested and reseeded in 60 mm dishes (5000 cells/dish) containing 2 ml of a 0.3% agarose suspension in DMEM plus 10%

fetal bovine serum and G418 (1mM). Plates were incubated at 37°C with 7% CO<sub>2</sub> for 3 weeks. The experiments were repeated in triplicate.

### **2.15 MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay for cell proliferation.**

HJC-2, BsB8, and U87-MG cells were plated in 6-well plates ( $2 \times 10^5$ ) and transfected either with pCGT7 vector alone or pCGT7-SF2/ASF expression plasmid. Seventy-two hours after transfection, cells were washed with PBS, and incubated with 1 ml of MTT working solution (DMEM with 0.5 mg/ml MTT) for 1 hour at 37 °C. At the end of the incubation, the converted dye was solubilized with 1ml acidic isopropanol (0.004 M HCL in isopropanol). The suspension was pipetted up and down to make sure that the converted dye dissolved. The dye solution was transferred into 1.5 ml eppendorf tube, and was centrifuged at 15,000 rpm for 5 min. The supernatant was transferred into a new eppendorf tube. Absorbance of the converted dye was measured at a wavelength of 570 nm with background subtraction at 650 nm.

### **2.16 Flow Cytometry analyses.**

Parental cells (HPRTase-deficient U87 MG and Primary Astrocytes) and hybrid clones ( HC-7 and HC-15) were plated on 60mm dishes as  $0.5 \times 10^6$  cells per dish and were grown in DMEM medium containing 10% FBS for 24 hours. Cells were fixed in 70% ethanol and stained with Guava Cell Cycle Reagent

(Guava Technologies, Cat.No. 4700-0160). Cell cycle analyses were performed by using Guava Easycycle Mini machine and software.

### **2.17 PEG-mediated cell fusion.**

The HPRTase-deficient U87-MG human glioblastoma cell line was used as fusion partner with primary human fetal astrocytes. PEG-mediated cell fusion was performed as described previously (Beggs et al., 1988) with some modifications. Equal numbers of U87-MG HPRT-deficient and primary astrocytes were mixed with serum-free medium in a 50 ml conical tube. Cell mixtures were spun down for 10 min at 500g. Supernatant was removed. Cell mixtures were resuspended in 1 ml of 50% polyethylene glycol 1500 (PEG 1500, Roche) by continuously stirring the cells with pipette tip. Cells were kept at 37 °C during the fusion. 1ml of pre-warmed medium was added to the fusion mixture by continuously stirring the cells as before over a period of 2 min. 5ml medium was added to the mixture, continuously stirring the cells for 3 min. Than 10 ml medium was added to the mixture and cells were incubated at 37 °C for 8 min. Cells were spun down and supernatant was discarded. Cells were re-suspended in DMEM containing 10% FBS and gentamycin.

### **2.18 Selection and identification of JCV-permissive cell lines.**

U87-MG and primary human fetal astrocytes (PHFA) were fused, and single cell clones were created as described above. A total of 32 single cell clones of fused cells were obtained. All single cell hybrid clones were infected with the Mad1

strain of JC Virus. Whole cell extracts were prepared at 8 days of infection. JCV-permissive clones were detected by western blot analyses of JCV early protein expression (Large T antigen) and late gene expression (VP1).

**CHAPTER 3 \***  
**GENERATION AND CHARACTERIZATION OF JCV-PERMISSIVE**  
**HYBRID CELL LINES**

**3.1 INTRODUCTION**

The lack of a convenient and reliable cell culture system has significantly hampered the ability to study the mechanisms involved in the life cycle of the human polyomavirus, JC virus (JCV), the causative agent of the demyelinating disease, progressive multifocal leukoencephalopathy (PML) (Khalili et al., 2003; Major and Ault, 1995). Replication of JCV in vitro takes place on an average of three or more weeks in culture (Radhakrishnan et al., 2003). As such, propagation of wild type and mutant viral stocks is a labor intensive and time-consuming undertaking. In comparison, the life cycle of the highly related simian virus 40 (SV40) is significantly shorter with death of host cells evident in as little as 24 h post-infection. The availability of several kidney epithelial cell lines, including CV-1, for cultivation and study of SV40 has helped to decipher the mechanisms involved in gene regulation, viral replication, and virus–host interaction. Unlike SV40, knowledge about the pathways involved in the life cycle of JCV remains more limited, mainly due to the absence of a continuous, well characterized cell line that permits efficient replication of JCV. In the laboratory, replication of JCV has been limited to primary cultures of human fetal glial cells, and thus far, no small animal model that supports viral replication has been identified (for review, see Khalili et al., 2003). The difficulties associated with

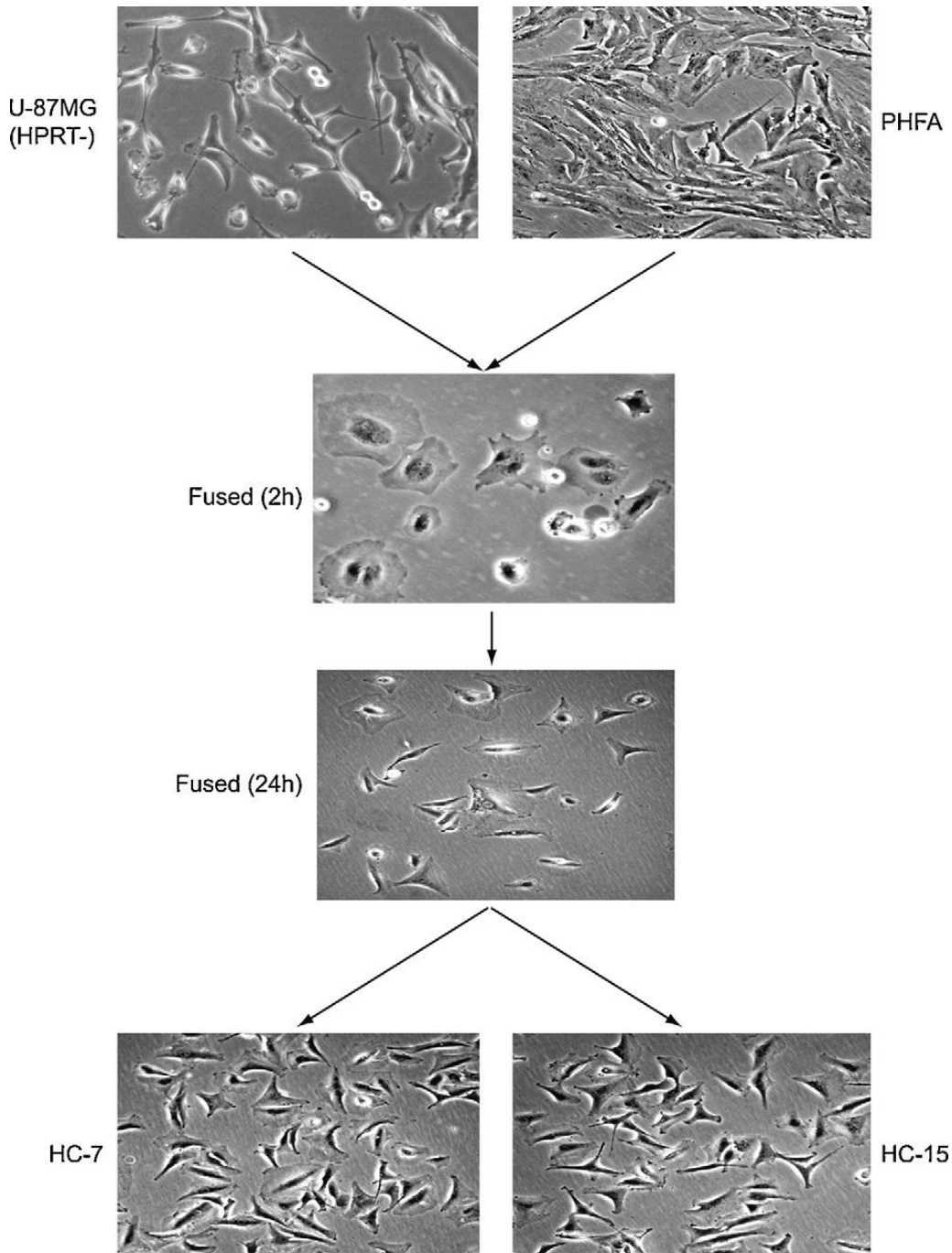
obtaining human fetal brain tissues for the preparation of primary cultures, which are costly, labor intensive, and can vary in purity from preparation to preparation, have prompted several investigators to develop cell lines including SVG and POJ that support the JCV infection cycle (Mandl et al., 1987; Major et al., 1985; Frye et al., 1997). However, the utility of these lines for studying initial events that stimulate viral gene expression and replication is limited as the culture systems are transformed with either SV40 (SVG-A) or JCV (POJ) genomes and constitutively express the T-antigens of these viruses. Thus, the constitutive presence of T-antigen in these cells bypasses the immediate early events in the JCV infection cycle including activation of the early promoter and expression of T-antigen. To overcome this issue, an alternative strategy of cell fusion was employed between permissive primary human fetal astrocytes and the non-permissive human glioblastoma cell line, U-87MG, and subsequently several hybrid cell lines were developed to study the JCV life cycle.

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## **3.2 RESULTS**

### **3.2.1 Generation of hybrid clones permissive for JCV replication using primary human fetal astrocytes (PHFA) and the glioblastoma cell line (U-87MG).**

In order to produce immortalized cell lines, which would efficiently and reproducibly support JCV replication in culture, PEG-mediated cell fusion of JCV permissive primary human fetal astrocytic cultures (PHFA) with non-permissive HPRT-deficient U-87MG human glioblastoma cell line was performed as described in Materials and Methods. Fusion efficiency was evaluated by the appearance of double-nucleated cells by phase contrast microscopy after fusion (Fig. 3.1). 32 hybrid clones were obtained and selected for further study. In order to identify individual hybrid clones which support viral gene expression and viral replication, cells were plated at passage 5 after cell fusion and infected with the Mad-1 strain of JCV. Strong expression of early and late genes large T antigen and VP-1, respectively was detected by Western blot at 8 days post infection in clones HC-7, HC-15 and HC-21 (data not shown). JCV proteins were also detected in clones HC-9, HC-12, HC-16 and HC-22, but expression levels were relatively lower. Clones HC-7 and HC-15 (Fig. 3.1) were selected for further analysis. Both hybrid clones supported JCV infection and are morphologically distinct from parental PHFA and U-87MG cells.

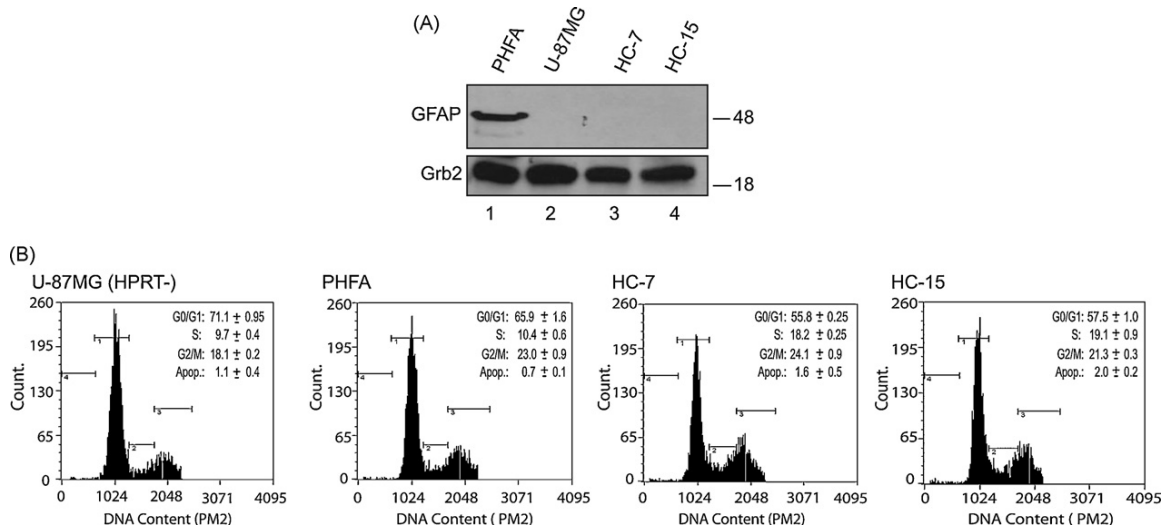


**Figure 3.1: Morphological features of parental (U-87MG-HPRT-deficient and PHFA) and hybrid clones (HC-7 and HC-15) before and after the cell fusion process.** Phase contrast images of hypoxanthine phosphoribosyl transferase

(HPRT)-deficient U-87MG cell line (U-87MG-HPRT-deficient) and PHFA before PEG-mediated fusion (upper panels). The HPRT-deficient U-87MG cell line was created from the parental U-87MG cell line by negative selection with 8-azaguanine (Sigma, #A5284) as described previously (Amano et al., 1974) and used as a fusion partner with PHFA cultures which were prepared and maintained as described previously (Radhakrishnan et al., 2003). Polyethylene glycol (PEG) mediated fusion between U-87MG-HPRT-deficient and PHFA cells was performed by combining equal numbers of U-87MG-HPRT-deficient and PHFA cells in serum free medium containing 50% PEG 1500 (PEG 1500, Roche) (Beggs et al., 1988). PEG-mediated fusion resulted in double-nucleated cells 2 h after fusion while at 24 h after fusion, most of the cells showed a single nucleated phenotype. Fused cells were grown in HAT selection medium for 72 h, and clonal cell lines were isolated by limiting dilution. Isolated clones HC-1 through HC-32 were further analyzed. Phase-contrast images of representative hybrid clones HC-7 and HC-15 at passage 6 show a phenotype distinct from their parental cells (bottom panels).

### **3.2.2 Analysis of the properties of JCV-permissive hybrid clones, HC-7 and HC-15.**

Examination by Western blot analysis of whole cell extracts of the astrocyte marker protein, glial fibrillary acidic protein (GFAP) revealed that, unlike PHFA, which expresses GFAP, neither of these proteins were detected in extracts from U-87MG cells, or in the hybrid cells, HC-7 and HC-15 (Fig. 3.2, Panel A). To compare the growth properties and DNA content of the hybrid cells with the parental cells, flow cytometry assay was performed. By determining the cell cycle profile of the clones, the distribution of the cells at G<sub>0</sub>/G<sub>1</sub>, S, and G<sub>2</sub>/M phases and the percentage of apoptotic cells were determined. As shown in Fig. 3.2 (Panel B), a modest decline in the cell distribution at G<sub>0</sub>/G<sub>1</sub> of the hybrid clones compared to their parental cells was noted. At the G<sub>2</sub>/M stage, the profile of the hybrid clones appears to be closer to PHFA than U-87MG (HPRT<sup>-</sup>) cells. Furthermore, a larger number of cells in S phase was noted in clonal cells than those in the parental cells as was observed, cells in S phase were detected in HC-15 (19.1%) versus PHFA (10.4%) and U-87MG (9.7%). These observations showed a subtle variation in the cell cycle distribution of the U-87MG and PHFA cells upon their fusion. However, no significant differences in the doubling times of the parental and the hybrid cells were observed, which ranged between 27 and 30 h (data not shown).



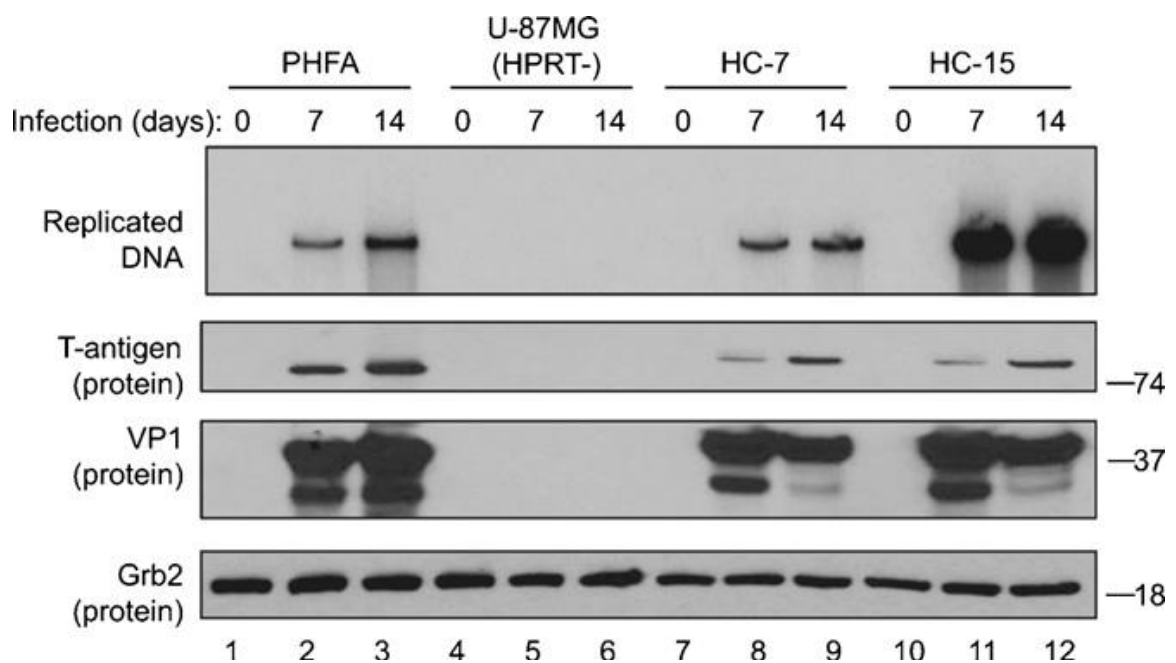
**Figure 3.2: Marker protein expression and flowcytometric analyses of hybrid clones.** (A) Western blot analysis of GFAP expression in parental and hybrid cells. Whole-cell extracts were prepared from U-87MG-HPRT-deficient, PHFA, HC-7, and HC-15 cells, resolved by SDS-PAGE, and transferred to nitrocellulose membranes. Expression of the cellular protein, GFAP, was determined using an anti-GFAP antibody. Grb2 was probed as a loading control. (B) Flow cytometric analysis of the parental (U-87MG-HPRT-deficient and PHFA) and hybrid clones (HC-7 and HC-15). Cells were fixed in 70% ethanol, stained with Guava Cell Cycle Reagent (Guava Technologies), and DNA content determined with the Guava EasyCycle Mini machine according to the manufacturer's instructions (Guava Cell Cycle Reagent, Guava Technologies).

### **3.2.3 HC-7 and HC-15 hybrid clones support JCV DNA replication and gene expression.**

In order to characterize JCV infection in the newly-generated HC-7 and HC-15 hybrid cell lines, we plated the cells at passage 10 after cell fusion and infected with the Mad-1 strain of JCV. Both parental cells, PHFA and U-87MG, were also included in the study group as positive and negative controls, respectively and we then asked whether they could support viral replication. Viral DNA was isolated at 7 and 14 days post-infection and analyzed by Southern blotting (Fig. 3.3). As expected, JCV replicates in PHFA cells and the level of replication approximately doubled by day 14 post-infection (compare lane 2 with lane 3). Hybrid clone 7 (HC-7) was also observed to support viral DNA replication at levels similar to those observed for PHFA parental lines (compare lanes 8 and 9 with lanes 2 and 3 respectively). However, another hybrid clone (HC-15) was found to be even more supportive of JCV replication both at 7 and 14 days post-infection. The level of viral DNA replication at both data points was higher than that observed for both hybrid clone HC-7 and parental PHFA cells (compare the lanes 11 and 12 with 8 and 9 and; with 2 and 3 respectively).

In parallel to the DNA samples analyzed for viral DNA replication as shown in Fig. 3.3, whole-cell extracts were prepared at 7 and 14 days post-infection and examined for viral gene products, including viral large T antigen and VP1 by Western blotting (Fig. 3.3). We observed a similar level of viral gene expression between parental cells (PHFA) and hybrid clones (compare lanes 2 and 3 with 8 and 9; and with 11 and 12, respectively). As expected, U-87MG

cells were negative for viral gene expression (lanes 4, 5 and 6). It was interesting to observe that even though the hybrid clones, HC-7 and HC-15, showed the ability to express a similar level of viral proteins compared to parental cells, HC-15 supported even more efficient viral DNA replication (Fig. 3.3, compare lanes 11 and 12 with lanes 8 and 9 respectively) than HC-7, suggesting a clonal variability between the established hybrid clones.



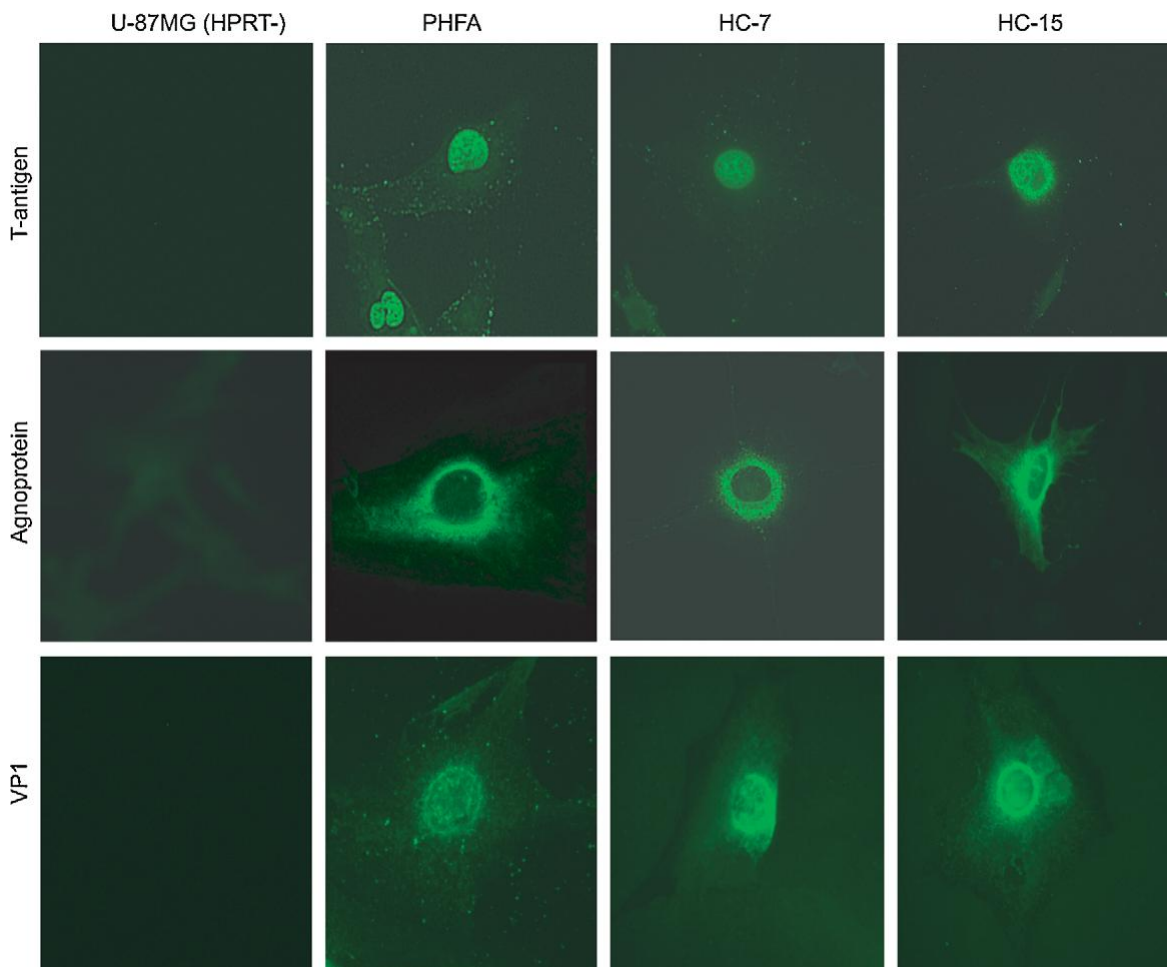
**Fig. 3.3: JCV replication and gene expression in hybrid clones (HC-7 and HC-15) and their parental cells.** Southern blot analysis of viral DNA isolated from infected cells (U-87MG-HPRT-deficient, PHFA, HC-7 and HC-15 cells) (upper panel). Cells were infected with the Mad-1 strain of JC virus and maintained in DMEM supplemented with 2% FBS and antibiotics at 37 °C until collected for protein extraction or DNA isolation. Transfection/infections were

performed as described previously (Sariyer et al., 2006). Low molecular weight viral DNA was purified from cells infected with the Mad-1 strain of JC virus at the indicated time points post-infection using a QIAprep-spin miniprep kit as described previously (Ziegler et al., 2004; Sariyer et al., 2008). Purified low molecular weight DNA was digested with BamHI and resolved by 1% agarose gel electrophoresis. The replicated viral genome was analyzed by Southern blotting using the entire Mad1 genome radiolabeled with [<sup>32</sup>P]-dCTP by Klenow reaction as probe as described previously (Sariyer et al., 2008). In parallel, Western blot analysis was performed on whole cell extracts prepared from U-87MG-HPRTdeficient, PHFA, HC-7 and HC-15 cells uninfected or infected with JC virus. Expression of JCV proteins was detected with antibodies recognizing large T-antigen (pAb416, Oncogene Research Products), and VP1 (pAb597, kindly provided by Walter Atwood). DNA or proteins isolated from uninfected cells served as negative controls (i.e. 0 days). Western blotting with anti-Grb2 antibody (Santa Cruz, C-19) demonstrates the equal loading of the samples (bottom panel).

#### **3.2.4 Localization of viral proteins in hybrid clones.**

Next, we sought to analyze the expression and localization of viral early and late gene products, including large T antigen, VP1 and agnoprotein, in HC-7, HC-15, PHFA and U-87 MG cells infected with JCV by immunocytochemistry (Fig. 3.4). Large T antigen and VP1 of polyomaviruses are known to be nuclear proteins, and we did not observe a deviation from this pattern for large T antigen

in both parental and hybrid lines. However, the distribution of VP1, on the other hand, appeared to be more perinuclear and partly cytoplasmic in hybrid clones HC-7 and HC-15 than parental PHFA. This clearly suggests a differential localization pattern of VP1 in both hybrid clones than parental cells (U-87MG HPRT (-) and PHFA), but the reason why it is so is currently unknown. As we previously reported (Safak et al., 2002; Sariyer et al., 2006), agnoprotein mostly localizes to the cytoplasm of both hybrid clones and parental cells with a high concentration accumulated the perinuclear area.

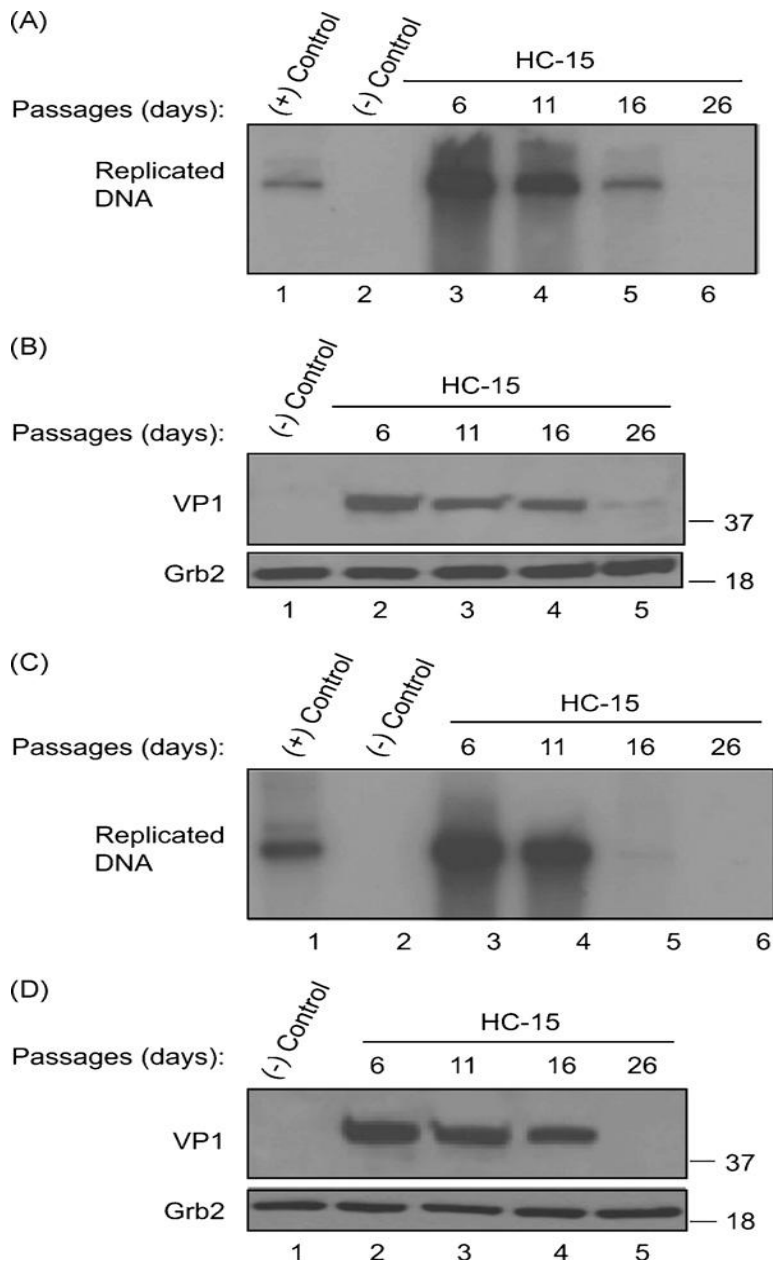


**Fig. 3.4: Immunocytochemistry for viral proteins in hybrid clones and parental cells infected with JCV.** U-87MG-HPRT-deficient, PHFA, and the hybrid clones HC-7 and HC-15 were infected with the Mad-1 strain of JCV, seeded onto chamber slides, and fixed with ice-cold acetone 8 days after infection. Immunocytochemistry to detect viral proteins was performed as described previously (Safak et al., 2002; Sariyer et al., 2006) with anti-large T-antigen (pAb416) and anti-VP1 (pAb597) monoclonal antibodies and anti agnoprotein polyclonal antibody (Del Valle et al., 2002) for 8 h. Cells were then washed with PBS, incubated with FITC-conjugated secondary antibodies for 2 h, then mounted with aqueous mounting medium and examined by indirect immunofluorescence microscopy. Results revealed expression of T-antigen, agnoprotein and VP1 in PHFA, HC-7, and HC-15 cells but not in U-87MG cells.

### **3.2.5 Hybrid clones gradually lose their permissibility for JCV infection at late passages.**

To test the longevity of the hybrid cells in supporting JCV replication, HC-15 cells at various passages ranging from 6 to 26 were infected with JCV, and replication of viral DNA and expression of the viral late protein, VP1, were determined at days 8 post-infection. As shown in Fig. 3.5, replication of JCV DNA and expression of VP1 was detected in passage 6, 11 and 16, but not 26, suggesting that the hybrid cells lose their ability to support JCV replication after repeated passaging in culture, i.e. >16 passages. Similar results were obtained

with HC-7 cells (data not shown). To rule out the involvement of receptors in the loss of permissiveness of high-passage hybrid clones, in the next series of experiments, cells were transfected with JCV DNA and replication of the DNA and expression of viral late proteins was determined. As shown in Fig. 3.5C, consistent with the findings from Fig. 3.5A, the replication of the viral genome in cells at passage 16 is barely detectable and completely abrogated by passage 26, supporting the notion that the loss of replication of the viral genome in high-passage hybrid lines is not due to the loss of receptors or any other barriers imposed during the viral entry process. In addition, results from Western blotting showed complete disappearance of viral late protein expression by passage 26. Altogether, these results show that fusion of PHFA and U-87MG produces cell lines that are permissive for JCV for several passages, at least up to 16.



**Figure 3.5: JCV replication is restricted at later passages of the hybrid clones.** Different passage numbers of the hybrid clone HC-15 (P6, P11, P16 and P26) were infected with the Mad-1 strain of JCV (Panels A & B) or transfected with viral DNA (Panels C & D) and DNA was isolated by QIAprep-spin miniprep kit at 8 days post infection. (A) Purified low molecular weight DNA was digested

with DpnI/BamHI and analyzed by Southern blotting to detect replicated viral DNA. In lane 1, 2 ng of Mad-1 genome digested with BamHI was loaded as a positive control. In lane 2, a DNA sample isolated from uninfected cells was processed as in lanes 3–6 and loaded as a negative control. (B) Whole cell extracts were prepared and analyzed for VP1 expression by Western blotting using anti-VP1 antibody. Reprobing of the blot with an anti-Grb2 antibody demonstrated equal loading of the samples. In lane 1 protein samples prepared from uninfected cells were loaded as a negative control. (C) Hybrid clones were transfected with the JCV Mad-1 genome using lipofectamine (GibcoBRL). Eight days post-transfection, viral DNA was isolated and analyzed by Southern blotting as described for Panel (A). (D) In parallel, whole-cell protein extracts were prepared and analyzed by Western blotting as described for Panel (B).

### **3.3 DISCUSSION**

After attachment of JC virus to the cell membrane and endocytosis, viral DNA enters the nucleus of the infected cells where it can be stimulated by specific transcription factors to produce the early gene product, T-antigen. T-antigen then recruits cellular proteins to stimulate viral DNA replication and late gene expression that results in the production of progeny capsids. In addition, several studies have ascribed a critical role for agnoprotein in successful virus production (Johannessen et al., 2008; Matoba et al., 2008; Sariyer et al., 2006, 2008). The major distinction between the more ubiquitous SV40 and JCV rests on the tissue specificity of JCV gene expression, which is more restricted. In addition, SV40 and JCV exhibit a high degree of species specificity with viral replication restricted to the natural host, i.e. simian and human.

One of the main drawbacks of hybridoma cell technology is the instability of the genome of hybrid clones (Duelli et al., 2005; Duelli and Lazebnik, 2007; Ganem et al., 2007; Hernandez et al., 1996). It is possible that the gradual loss of JCV replication at the higher passages of the hybrid lines reflects genomic instability of the hybrid clones. It should be noted that JCV proteins, T-antigen and agnoprotein, have been shown to interfere with DNA repair machinery and to induce chromosomal instability (White and Khalili, 2005; Khalili et al., 2003, 2005). Thus, the sensitivity of the hybrid clones to chromosomal damage may be enhanced upon JCV infection, leading to the loss of genes upon repeated passaging that are responsible for expression of specific factors that potentiate the JCV genome. Another possibility for restricted JCV replication at higher

passages, though not mutually exclusive, may come from the activation of cell-type specific inhibitors of viral gene expression. This possibility is consistent with earlier fusion studies in which mouse fibroblasts were fused with transformed hamster glial cells and the expression of JCV large T-antigen was lost in the hybrid cells (Beggs et al., 1988, 1990). These observations suggest that expression of negative regulatory protein(s) in the hybrid cells can suppress JCV gene expression and replication. Further studies are required to understand the mechanism for extinction of JCV in the hybrid cells.

In addition to glial origin cells, including primary human fetal astrocytes and the T-antigen transformed glial origin cell lines POJ and SVG described above, some strains of JCV have been able to grow in non-glial cell culture systems (Khalili et al., 2003). These include primary cultures of Schwann cells and neuroblastoma cell lines, as well as kidney epithelial cells (Akatani et al., 1994; Assouline and Major, 1991; Hara et al., 1998). In addition, JCV has been shown to replicate in hemotopoietic progenitor cells, B lymphocytes, and tonsillar stromal cells with varying efficiency (Monaco et al., 1996). Of note, JCV infection does not appear to be restricted at the level of binding and cell entry (Ashok and Atwood, 2006). The cell lines capable of supporting JCV infection have all been of human origin, with a few notable exceptions being of simian origin (Hara et al., 1998). These findings are consistent with the high degree of species specificity seen with the polyomavirus family and also with the natural history of JCV infection in humans. As an alternative to primary cultures of human fetal astrocytes, hybrid human cell lines were produced by fusing primary human fetal

astrocytes with the human malignant glioblastoma cell line, U-87MG by negative selection using HPRT. Several JCV-permissive hybrid cell lines were identified and two of these clones were examined in detail with respect to hybrid properties and viral propagation. Both hybrid cell lines and PHFA cells were infected and compared with respect to viral transcription and replication. It was demonstrated that the newly-generated hybrid cell lines support JCV replication and viral protein production at multiple passages. The hybrid lines supported JCV replication and propagation at early passages but lost that ability at late passages, suggesting that these cells may provide a system for identifying positive and negative regulators of viral transcription and replication. In addition, these newly-generated hybrid cell lines may serve as a good tissue culture model system for JCV replication and propagation and may help us develop more effective strategies to prevent JCV infection in glial cells.

**CHAPTER 4**

**STUDY OF THE IMPACT OF SF2/ASF ON JCV REPLICATION AND GENE  
EXPRESSION IN GLIAL CELLS**

**4.1 INTRODUCTION**

JCV is a human polyomavirus that infects greater than 80% of the human population during childhood (Weber, 2008; Imperiale and Major, 2007). Replication of the neurotropic strain of JCV in glial cells causes the fatal demyelinating disease of the central nervous system, progressive multifocal leukoencephalopathy (PML), which is seen in patients with underlying immunosuppressive conditions, which notably occurs in AIDS patients (Berger and Concha, 1995; Safak et al., 2005; Miller et al., 1982; Eng et al., 2006). Recently, several cases of PML have been reported in patients under treatment with immunomodulatory drugs including natalizumab, rituximab, and efalizumab, indicating that alterations in immune status may lead to reactivation of latent and/or passing virus in human brain (Sandborn et al., 2005; Langer-Gould et al., 2005; Kleinschmidt-DeMasters et al., 2005; Carson et al., 2009). Like other polyomaviruses, the JCV genome is composed of double-stranded circular DNA of approximately 5 kb in size with a bi-directional non-coding control region that is located between the early and late coding sequences. The early coding region is responsible for the expression of large T-antigen, small t-antigen, and a group of T' proteins, all of which are produced upon alternative splicing of a specific primary transcript (Trowbridge P.W., Frisque, 1995). Similarly, alternative

splicing of the late transcript results in production of the viral capsid proteins VP1, VP2, and VP3, all of which are important for completion of the viral lytic cycle and formation of viral particles. Processing of both early and late primary transcripts requires participation of splicing factors, including SF2/ASF, a ubiquitous factor which plays a pivotal role in alternative and constitutive splicing of precursor mRNAs of mammalian cells (Licatalosi and Darnell, 2010; Manley and Tacke, 1996; Graveley, 2000; Sanford et al., 2005; Caceres et al., 1997). SF2/ASF was first discovered as a cell type-specific regulator of another well-studied member of the polyomavirus family, SV40, based on its ability to modulate splicing of the viral early gene, thus effecting the expression of large T-antigen and small t-antigen expression at the post transcriptional level (Ge and Manley, 1990; Wang and Manley, 1995). The non-coding control region of the neurotropic strain of JCV, Mad-1, is composed of two 98 bp tandem repeats that have cell type-specific characteristics, and its activation primarily occurs in glial cells such as oligodendrocytes and astrocytes (Safak et al., 2005; Miller et al., 1982). Earlier results from our laboratory and others led to the identification of several constitutive and inducible cellular factors with the ability to positively and negatively control JCV gene transcription (White et al., 2006 and 2009). These observations led us to postulate that transcription of the JCV promoter is controlled by a group of transcription factors that universally silence expression of the viral genome under normal conditions and that alter the level of their expression and/or activities under certain physiological conditions, such as immunosuppression, thus providing an opportunity for the virus to replicate in the

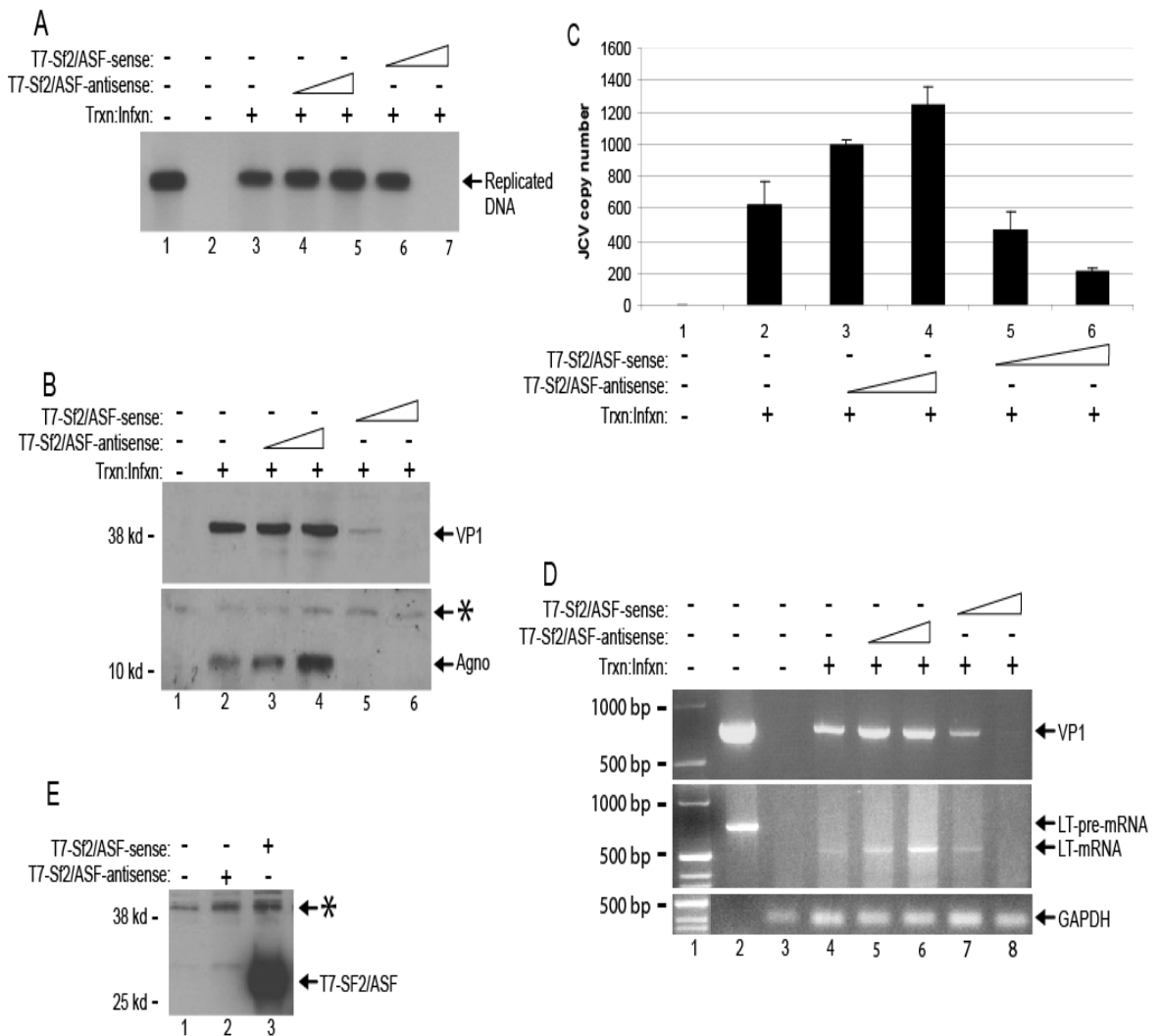
permissive cells of the CNS. Here, we examined the possible effect of SF2/ASF on the regulation of JCV gene expression and viral replication in glial cells. Our results show that while SF2/ASF plays a role, similar to that seen in SV40, in splicing viral transcripts, it has a profound impact on transcription of the viral genome and replication of JCV in glial cells. Our results show that overexpression of SF2/ASF suppresses JCV gene transcription in human glial cells. Accordingly, suppression of SF2/ASF enhances the level of viral replication in astrocytic cells. These observations provide the first evidence for regulation of promoter activity by the splicing factor, SF2/ASF, and shed new light into regulation of JCV gene transcription and replication.

## **4.2 RESULTS**

### **4.2.1 SF2/ASF suppresses replication of JCV in glial cells.**

To investigate the effect of SF2/ASF on replication of JCV, primary human fetal astrocytes, PHFA, were transfected with JCV Mad-1 DNA, either alone or together with a plasmid expressing SF2/ASF in the sense or antisense orientation. Overexpression of SF2/ASF had a negative effect on replication of JCV DNA (Fig. 4.1A) and production of the viral proteins, VP1 and agnoprotein (Fig. 4.1B). In the antisense orientation, expression of SF2/ASF showed no inhibitory effect, suggesting that the overexpression of SF2/ASF, as shown in Fig. 4.1E, is required for the observed suppression. Expression of SF2/ASF in PHFA also decreased the copy number of the virus during the course of

infection, indicating that the decrease in viral gene expression by SF2/ASF has an impact on the viral lytic cycle and its propagation (Fig. 4.1C). In cells expressing SF2/ASF in the antisense orientation, more virus was detected in the culture media. Examination of viral transcripts by RT-PCR further established the inhibitory effect of SF2/ASF on expression of the JCV early and late genome (Fig. 4.1D).



**Figure 4.1: Overexpression of SF2 inhibits JCV propagation in PHFA cells.**

A. Southern blot analyses of JCV-infected PHFA cells. In lane 1, 2 ng of linearized Mad-1 genome was used as a positive control. In lane 2, DNA samples from uninfected cells were loaded as negative controls. B. Western blot analyses of whole-cell extracts prepared in parallel to DNA samples in panel A, using specific antibodies against VP1 and agnoprotein. In lane 1, whole-cell extracts from uninfected cells as a negative control. The asterisk denotes a nonspecific band recognized by agnoprotein antibody and used as loading control. C. Q-PCR analyses of viral particles in JCV infected-cell culture medium. D. RT-PCR analyses of JCV early (T-Ag) and late (VP1) gene products in JCV-infected PHFA cells. In lane 1, Kb ladder was loaded as a molecular weight marker. In lane 2, the JCV Mad-1 genome was used as a positive control. E. Expression of T7-SF2/ASF in PHFA cells. The asterisk represents a nonspecific band recognized by T7 antibody.

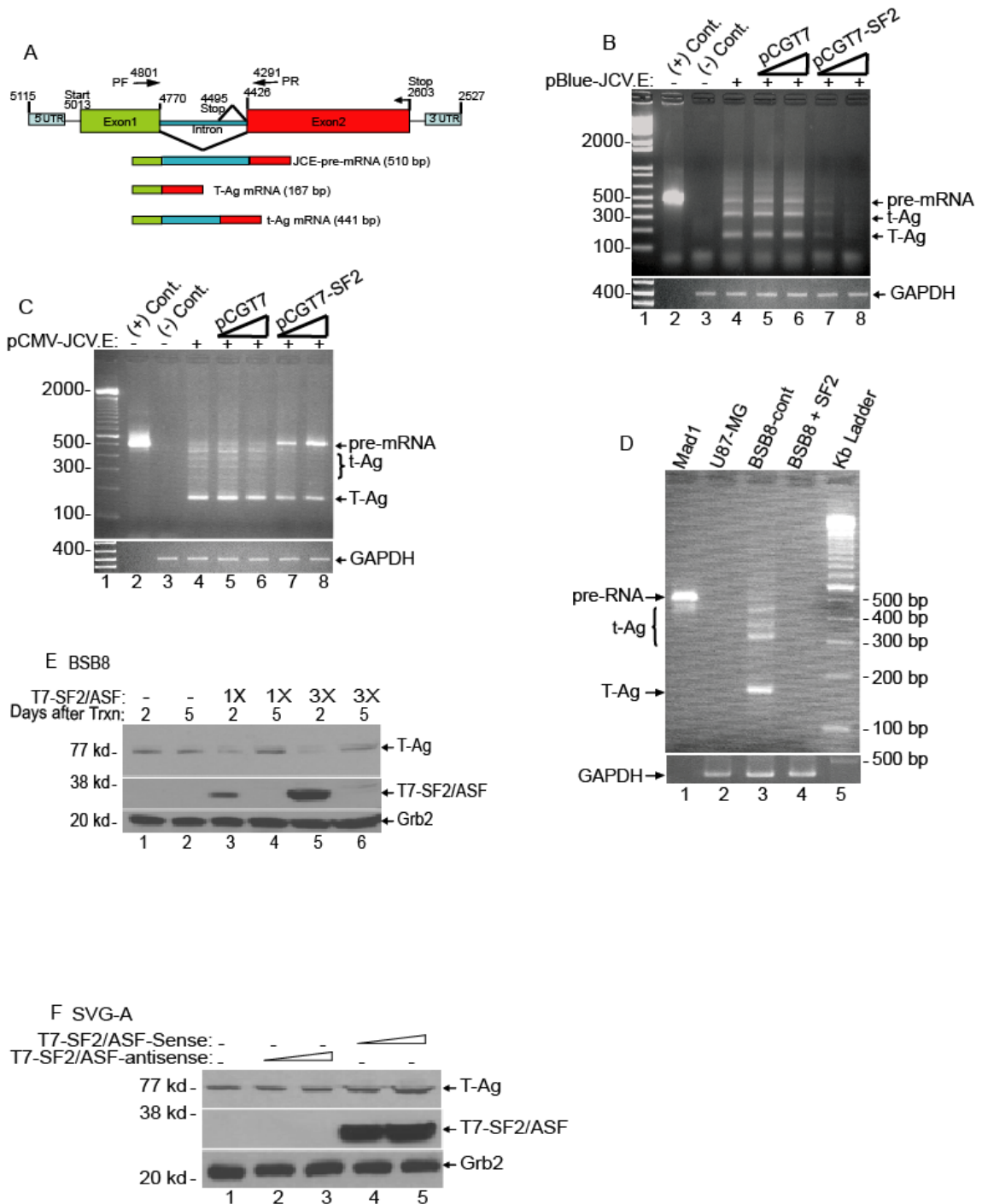
**4.2.2 Effect of SF2/ASF on splicing of the JCV transcript.**

To further demonstrate the effect of SF2/ASF on splicing of the JCV transcript, a plasmid containing the JCV early promoter expressing the early transcripts was introduced into PHFA along with a plasmid expressing SF2 (pCGT7-SF2) or the empty vector (pCGT7). RNA analysis was carried out by RT-PCR using primers that amplify alternatively spliced forms of JCV early transcripts (Fig. 4.2A). Interestingly, results showed that ectopic expression of SF2 significantly diminished the level of the viral transcript, suggesting that

SF2/ASF may have a negative effect on transcription of the JCV promoter (Fig. 4.2B). To further investigate the specificity of this observation, PHFA were transfected with a plasmid containing the CMV promoter expressing the JCV early transcript. As shown in Fig. 4.2C, expression of SF2/ASF had no effect on transcription of JCV-early genes induced by CMV promoter and that JCV early RNA was synthesized and in cells with overexpression of SF2, unspliced viral RNAs accumulated, and splicing of the primary transcripts for production of small t-antigen RNA was severely suppressed. These observations suggest that similar to its effect on splicing of SV40 (Wang and Manley, 1995), overexpressed SF2/ASF can also impair JCV early RNA splicing.

The unanticipated observation of the suppression of JCV promoter activity by SF2/ASF in transfected cells prompted us to determine whether transcription of the integrated copy of the JCV genome in cellular chromosomes can be influenced by SF2/ASF. To this end, we utilized a transgenic mouse cell line containing the JCV early promoter expressing the early genome. Results from RNA analysis by RT-PCR showed that ectopic expression of SF2/ASF severely suppresses production of JCV RNA but not control GAPDH transcripts (Fig. 4.2D). Examination of early gene expression at the protein level corroborated results from RNA analysis showing that production of SF2/ASF inhibits accumulation of the viral early protein, T-antigen, in the cells (Fig. 4.2E). Again, this observation was specific to JCV gene expression as evaluation of SV40 early protein production in SVGA, which contains an integrated copy of the SV40

early region, showed no effect of SF2/ASF on SV40 T-antigen expression (Fig. 4.2F).



**Figure 4.2: Effect of SF2/ASF on JCV early gene splicing in glial cells. A.**

Schematic structure of JCV early region unspliced and spliced RNAs and the

size of the expected amplification products with a primer set (PF and PR) used for the amplification of the JCV gene products in panels B, C, and D. B. SF2/ASF inhibits expression of JCV early region driven by its own promoter. pBlueScript-JCV-early reporting JCV early region under JCV promoter (pBlue-JCV.E), was transiently transfected into PHFA cells in the presence or absence of an SF2/ASF expression plasmid. Total RNA was extracted and used for cDNA synthesis by reverse transcriptase reaction. JCV-early region gene products (pre-mRNA, t-Ag and T-Ag) were amplified and separated on a 3% agarose gel and stained with ethidium bromide. Lane 2 was pBlue-JCV.E plasmid DNA amplified as positive control. Lane 3 was untransfected PHFA cell extracts used as a negative control. All of the bands reflecting the amplification products were separately cut from the gels and purified, and RNA identities were confirmed by sequencing. GAPDH was also amplified in the same cDNA samples as input control. C. SF2/ASF inhibits splicing of the JCV early region driven by the CMV promoter. PHFA cells were transiently-transfected with pCMV-JCV.E in the presence or absence of an SF2/ASF expression plasmid. Total RNA was extracted and processed for reverse transcriptase reaction as described in panel B. In lane 2, pCMV-JCV.Early plasmid DNA was amplified and loaded as a positive control for the primary transcript. Lane 3 shows untransfected PHFA cell extracts and was used as a negative control for amplification. D. BsB8 cells were transfected with either vector alone (lane 3) or with SF2/ASF (lane 4) expression plasmids. Expression of JCV-Early gene products were analyzed by RT-PCR as described in panels B and C. U-87 MG cells (lane 2) were also processed as

lanes 3 and 4, and were used as negative controls for amplification. E. Over-expression of SF2/ASF inhibits T-Ag expression in BsB8 cells. BsB8 cells were plated in 6-well dishes and transfected with the SF2/ASF expression plasmid in increasing concentrations (1X and 3X). Whole cell extracts were prepared at the 2nd and 5th days post-transfection and processed by Western blotting using specific antibodies against T-Ag and T7-tagged SF2 proteins. Grb2 was probed as a loading control after stripping of the same primary membranes used for Large T antigen and T7 protein detections. F. SF2/ASF shows no inhibitory effect on SV40 Large T antigen expression in SVG-A cells. SVG-A cells were plated in 6-well plates and transfected either with SF2/ASF-sense or with SF2/ASF-antisense expression plasmids. T-Ag and t-Ag expression were detected by Western blotting. Expression of transfected SF2/ASF and the cellular protein Grb2 was detected in the same blots by using T7-specific and Grb2-specific antibodies.

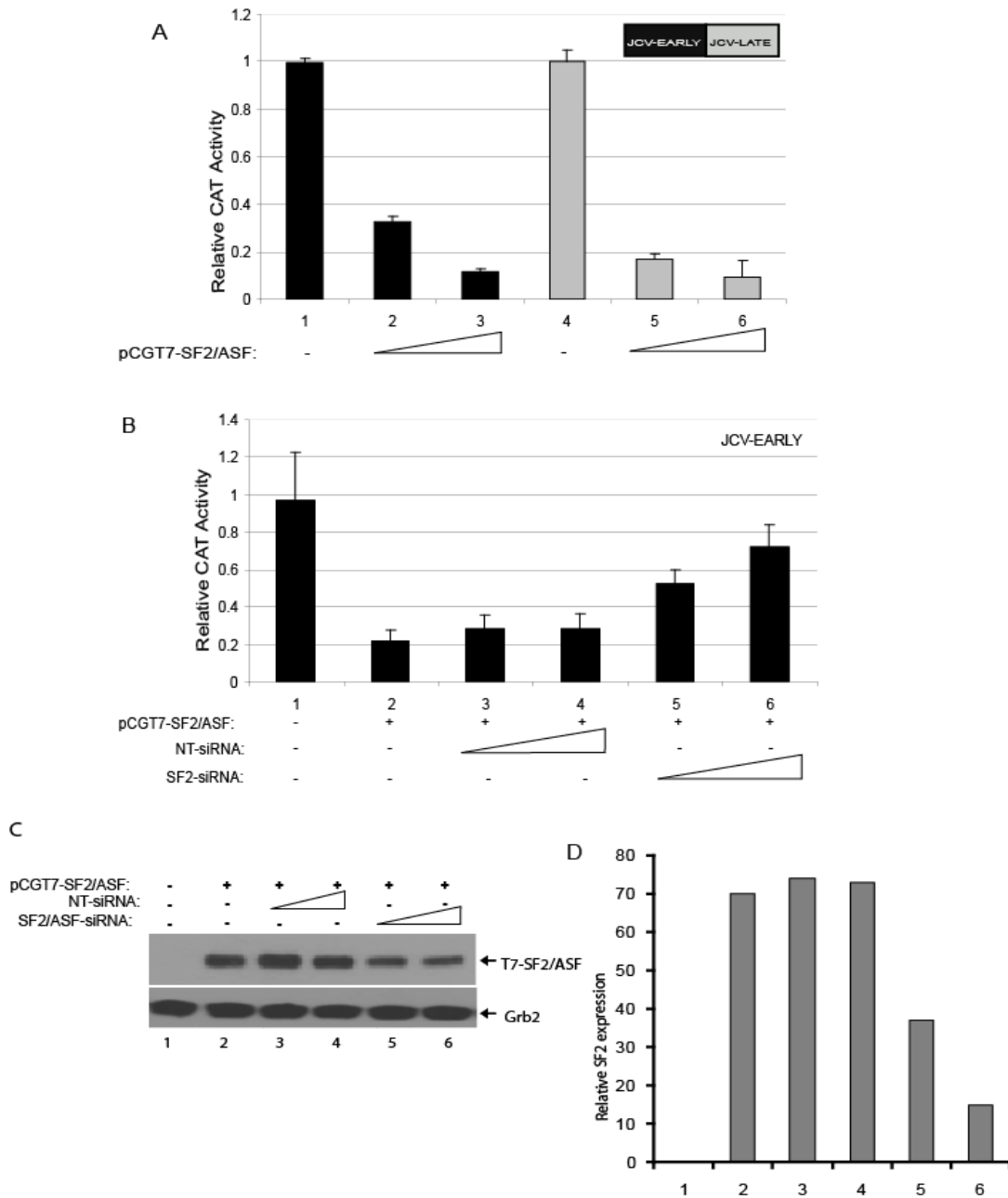
#### **4.2.3 Inhibition of JCV promoter activity by SF2/ASF.**

The data above suggest that SF2/ASF might suppress JCV gene expression at the transcriptional level rather than the posttranscriptional level. Therefore, we sought to analyze the effect of SF2/ASF on JCV early and late promoter activities in PHFA cells by CAT-reporter assays. PHFA cells were transiently transfected with Mad1-early or -late reporter constructs in the presence or absence of increasing concentrations of SF2/ASF expression plasmid. As hypothesized, over-expression of SF2/ASF strongly down-regulated

JCV-early and -late basal promoter activities in a dose-dependent manner (Fig. 4.3A).

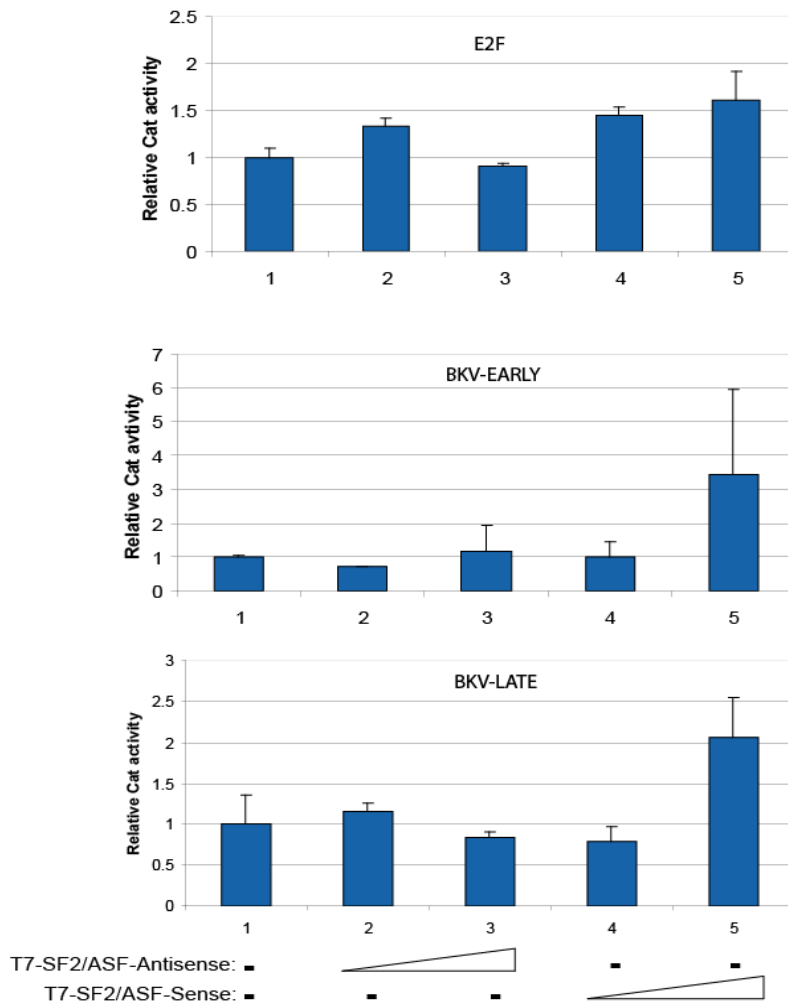
Next, we asked whether SF2-mediated inhibition of JCV promoter activity could be recovered using an SF2-specific siRNA (Fig. 4.3B). The JCV-early gene reporter plasmid was introduced into the PHFA cells with an SF2/ASF expression plasmid either alone or in combination with a non-targeting siRNA or an SF2-specific siRNA by co-transfection. Though SF2/ASF over-expression inhibited JCV early promoter activity ~5 fold, this inhibition was partially rescued by SF2-specific siRNA but not by a non-targeting siRNA. Downregulation of SF2/ASF by a specific siRNA was validated by western blotting in the same samples used for CAT reactions (Fig. 4.3C).

Reporter gene analyses indicated that overexpression of SF2/ASF in glial cells strongly inhibited JCV transcription (Fig. 3.3). In order to rule out the specificity of JCV transcriptional suppression by SF2/ASF, the effect of SF2/ASF expression on E2F-1 (a transcription factor) and BK virus (another member of human polyomaviruses which shares 75 % homology with JC virus) transcription was also tested in a similar manner as for JCV transcription. In contrast to the reporter assays from JCV, over-expression of SF2/ASF did not show any inhibitory effect on the E2F-1 promoter or the BKV-early and late promoters (Fig. 4.4), indicating that the negative regulation of JCV gene expression by SF2/ASF was specific to the JCV promoter.



**Figure 4.3: SF2/ASF inhibits JCV-early (JCV<sub>E</sub>) and -late (JCV<sub>L</sub>) transcription in PHFA cells.** A. JCV<sub>E</sub> (black bars) or JCV<sub>L</sub> (grey bars) reporter plasmids were transiently transfected into PHFA cells either alone or in combination with an SF2/ASF expression plasmid. At 48 h post-transfection, cells were harvested and CAT activities were determined. B. SF2/ASF-mediated inhibition of JCV-early

transcription can be rescued by a specific siRNA against SF2/ASF. JCV<sub>E</sub> reporter plasmid was transiently transfected into PHFA cells either alone or in combination with an SF2/ASF expression plasmid (lanes 2-6). At 12 h post-transfection, cells were transfected either with a non-targeting siRNA or with a siRNA specific to SF2/ASF. C. Western blot analyses of the same extracts used for reporter assays in panel B, using specific antibodies to T7-SF2/ASF and Grab2. D. Band intensities of SF2/ASF expression from panel E were quantified and shown as a bar graph.

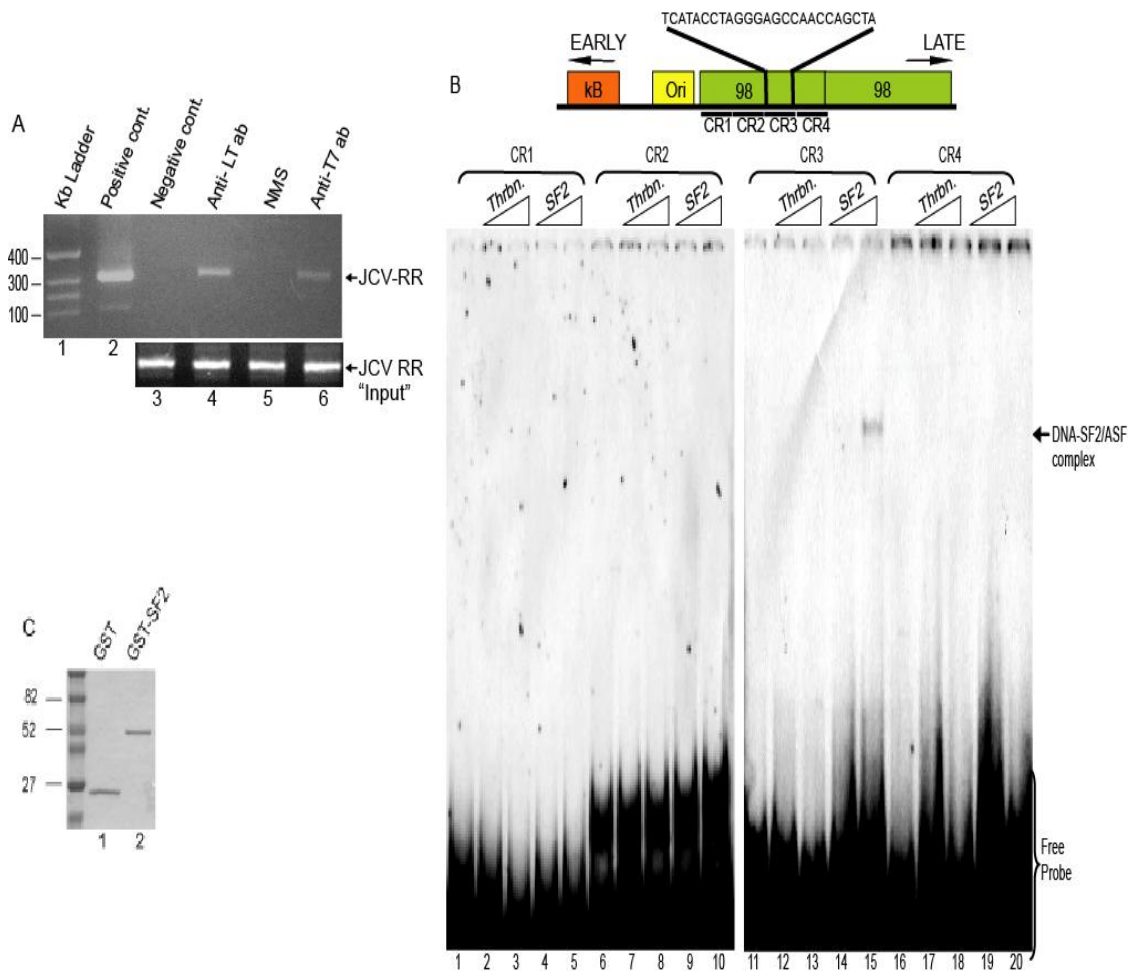


**Figure 4.4: Effect of SF2/ASF on E2F, BKV -early, and -late promoters.** CAT reporter plasmid (4µg/dish) containing promoter sequences of E2F, BKV-early and -late promoters were transiently transfected into PHFA cells in the presence or absence of pCGT7-SF2/ASF-antisense and -sense expression plasmids (1µg or 2µg/dish). At 48 h post-transfection, cells were harvested and CAT enzyme activities were determined.

#### **4.2.4 SF2/ASF binds to the JCV regulatory region in vivo and in vitro.**

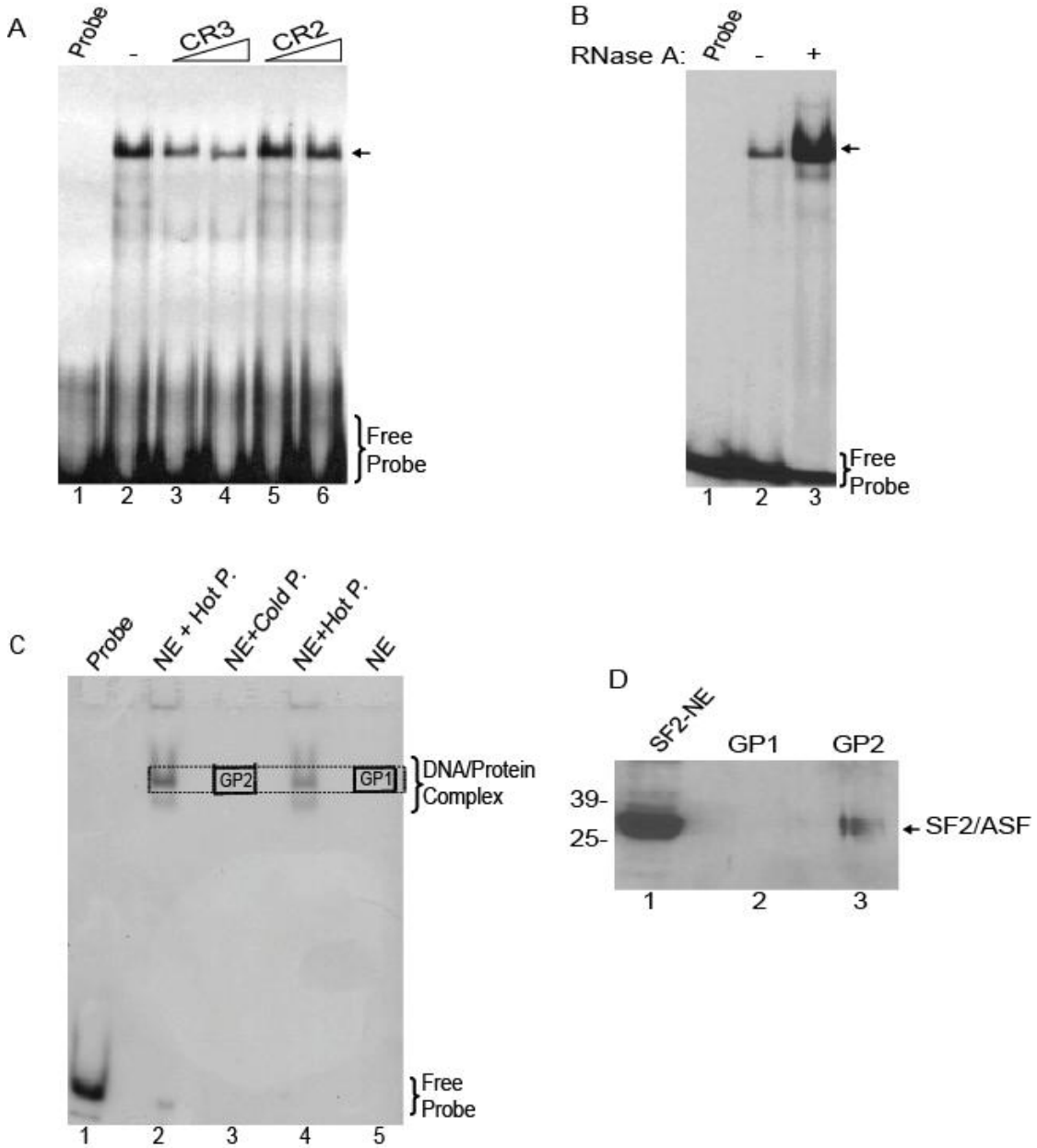
To better understand the events associated with SF2/ASF suppression of JCV promoter activity, we designed a series of experiments to assess the ability of SF2/ASF to bind to the JCV promoter sequence. ChIP analysis of BsB8 cells demonstrated an association of SF2/ASF with the JCV regulatory region. The DNA corresponding to the 98 bp tandem sequence of the JCV promoter was immunoprecipitated with antibody that pulled down SF2/ASF, suggesting a potential association of SF2/ASF with JCV DNA in BsB8 cells (Fig. 4.5A). To more precisely identify the region within the viral promoter that is a binding site for SF2/ASF, we used a series of oligonucleotides, named CR1, CR2, CR3, and CR4, (Fig. 4.5B), as probes in gel shift assays. To avoid any problems associated with non-specific binding, we used highly purified bacterially produced SF2/ASF in the binding reaction. As shown in Fig. 4.5B only CR3, which corresponds to the central region of the 98 bp repeat, showed binding activity to SF2/ASF. Next, we performed a series of gel shift experiments using nuclear extracts from PHFA and CR3 DNA probe to assess SF2/ASF interaction with the

JCV DNA sequence. Results from gel shift experiments showed the detection of a major DNA-protein complex whose formation was disturbed by unlabeled CR3 DNA competitor but not by CR2 under similar conditions, pointing to the specificity of the DNA-protein complex (Fig. 4.6A). The addition of RNase to the binding reaction enhanced the CR3-protein complex, suggesting that removal of the RNA molecules in the extract facilitates the interaction of the CR3 binding protein to the CR3 DNA probe (Fig. 4.6B). This is an interesting observation as SF2/ASF is a known RNA binding protein. To investigate the identity of the protein associated with CR3, a CR3-associated complex in PHFA was fractionated on a preparative native polyacrylamide gel (Fig. 4.6C) and the complex was analyzed by SDS-PAGE followed by Western blot using anti-SF2/ASF antibody. As seen in Fig. 4.6D, a sample corresponding to the CR3 nucleoprotein complex named GP2 reacted with anti-SF2/ASF antibody, indicative of SF2/ASF association with CR3 nucleoprotein complex.



**Figure 4.5: SF2/ASF directly targets the JCV regulatory region in vivo and in vitro.** A. BsB8 cells were transfected with T7-SF2/ASF expression vector, cross-linked, and analyzed by ChIP assay using antibodies to Large T antigen (lane 4), Normal mouse serum (lane 5), T7-tagged SF2/ASF (lane 6), and no antibody (lane 3). In lane 2, the JCV Mad-1 genomic DNA was used as a positive control. B. Gel shift analysis of JCV oligonucleotides spanning the 98-bp repeated region of the viral promoter with recombinant SF2/ASF. Four oligonucleotide probes (CR1, CR2, CR3, CR4), and the JCV regulatory region

with early and late orientations are schematized at the top of the panel. C. Coomassie blue staining of GST (lane 1) and GST-SF2 (lane 2).



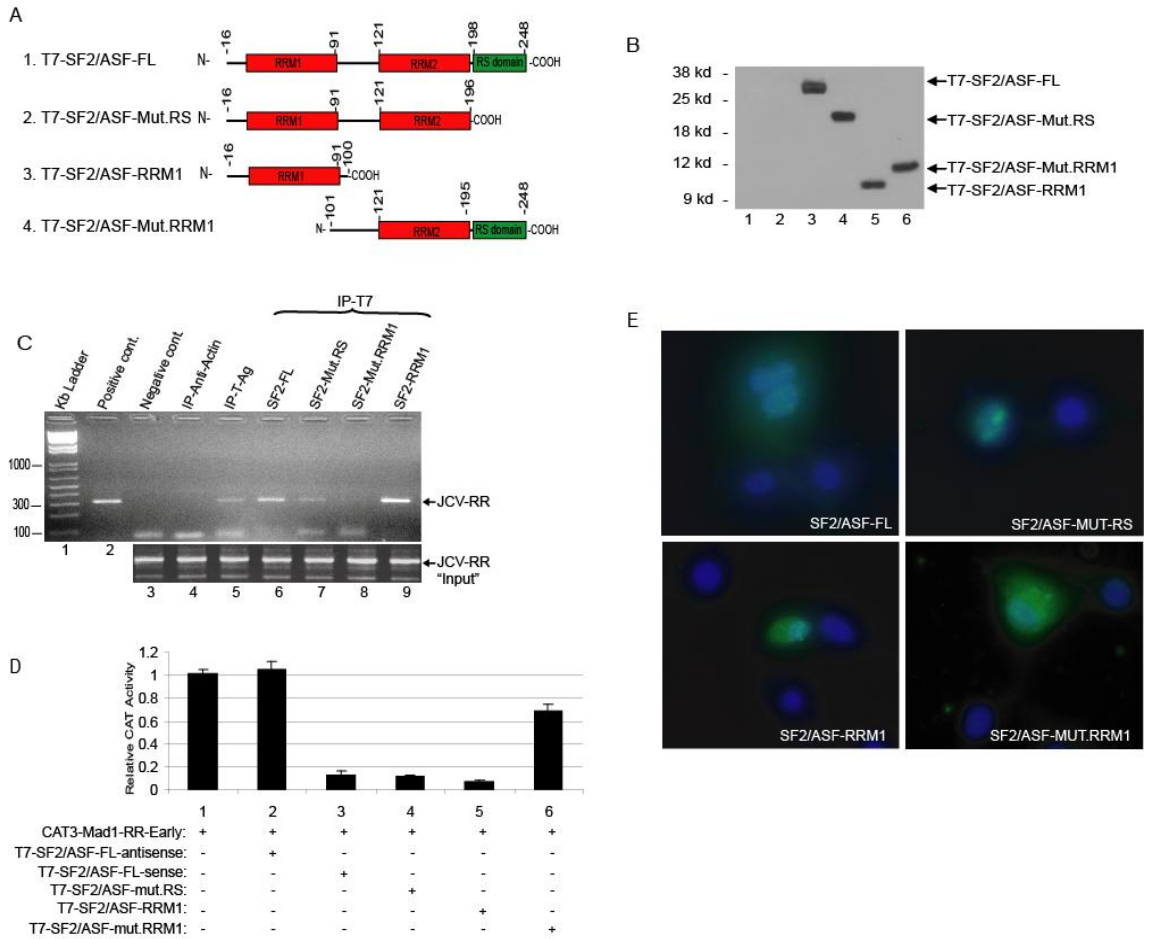
**Figure 4.6: Characterization of the interaction between SF2/ASF and the JCV regulatory region by gel shift assays. A. Competition analyses of the**

CR3 oligonucleotide of the JCV regulatory region. Nuclear extracts from PHFA cells incubated with end-labeled double stranded CR3 oligonucleotide probe in the presence of cold CR3 (lanes 3 and 4) and CR2 (lanes 5 and 6) oligonucleotides. B. Presence of RNA in nuclear extracts influences the binding pattern to CR3 oligonucleotide. Nuclear extracts from PHFA cells incubated with end-labeled double stranded CR3 oligonucleotide probe in the presence or absence of RNase A (lanes 2 and 3, respectively). C. Gel shift analysis of JCV CR3 oligonucleotide in primary human fetal astrocyte extracts. End-labeled (lanes 2 and 4) or cold (lane 3) CR3 oligonucleotide incubated with PHFA nuclear extracts. Radioactively-labeled CR3/NP complexes (lanes 2 and 4) were used as a reference to label and to cut the gel pieces containing the CR3/NP complexes in cold oligonucleotide reaction (lane 3, GP2) and in the nuclear extract control lane (lane 5, GP1). Dotted-lines indicate the vertical and horizontal lining of the expected running pattern of CR3/NP complexes. Solid boxes indicate the gel pieces (GP1, GP2) that were cut from the native gel. D. Nucleoprotein complexes in native gel pieces (GP1 and GP2) from panel C were denatured, resolved by SDS-PAGE, and were analyzed by Western blotting, using a specific anti-SF2/ASF antibody. In lane 1, nuclear extract from PHFA cells was loaded as a positive control.

#### **4.2.5 The RRM1 domain of SF2/ASF interacts with the JCV promoter DNA sequence and inhibits viral gene transcription.**

SF2/ASF has a modular structure consisting of two copies of the N-terminus RNA recognition motif, RRM1 and RRM2, followed by an arginine-serine rich, RS, domain in its C-terminus (Caceres et al., 1997; Zuo and Manley, 1993) (Fig. 4.7A). To assess the importance of these domains in the control of JCV gene transcription, we created a series of deletion mutants of SF2/ASF and examined their expression in PHFA (Fig. 4.7A and B). The association of each mutant protein with the JCV promoter was investigated by ChIP assay. and the results showed that mutant protein with no RRM1 showed no binding activity to JCV DNA. On the other hand, mutant protein containing only RRM1 exhibited strong binding activity to the JCV promoter (Fig. 4.7C). Results from transcription experiments showed that expression of RRM1 in PHFA is sufficient for suppression of the JCV promoter by SF2/ASF protein (Fig. 4.7D).

Previous studies have indicated that SF2/ASF mainly localizes to the nucleus in various cell types and shuttles between the nucleus and cytoplasm (Caceres et al., 1997; Zuo and Manley, 1993; Cazalla et al., 2002). In accordance with this observation, analyses of the sub-cellular localization of SF2/ASF in PHFA cultures by immunocytochemistry also showed nuclear localization with a speckled appearance (Fig. 4.7E). Interestingly, the SF2-Mut-RRM1 construct, which retains the RRM2 and RS domains, was localized in both of the nuclear and cytoplasmic compartments. On the other hand, the RRM1 domain alone was exclusively localized to the nucleus in PHFA cells.

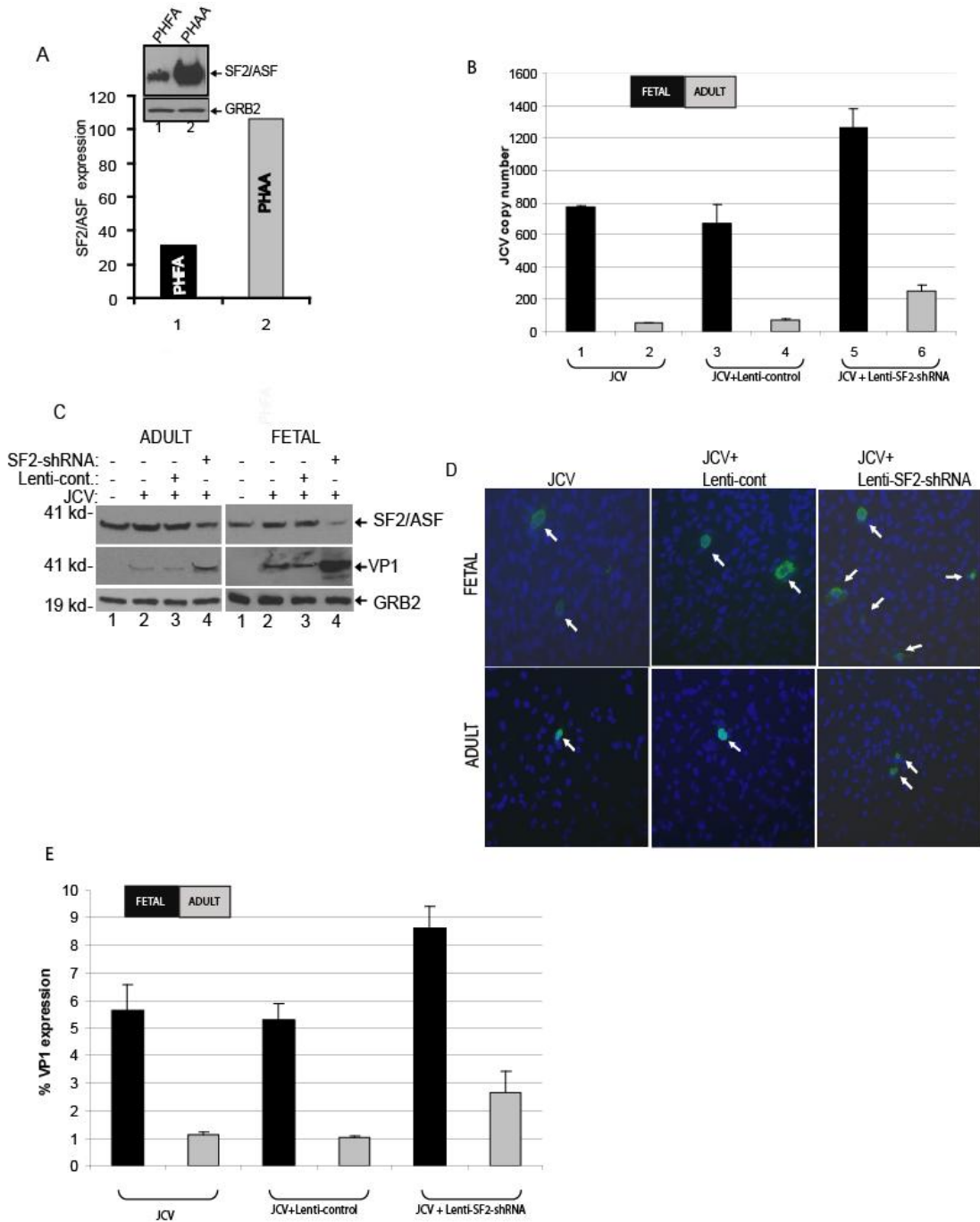


**Figure 4.7: “RRM1” domain of SF2/ASF is required for the inhibition of the JCV-Early promoter.** A. Schematic representation of SF2/ASF-FL (1) and its functional domain mutations, Mut.RS (2), RRM1-alone (3), and Mut.RRM1 (4). B. Expression of T7-SF2/ASF-FL and truncated forms of SF2/ASF. C. BsB8 cells were transfected with T7-SF2/ASF constructs, as schematized in panel A, cross-linked, and analyzed by ChIP assay using antibodies to actin (lane 4), T-Ag (lane 5), T7 (lanes 6 to 9), and no antibody (lane 3). D. JCV<sub>E</sub> reporter plasmid transiently transfected into PHFA cells either alone (lane 1) or in combination with T7-SF2/ASF-FL-antisense (lane 2), T7-SF2/ASF-FL-sense (lane 3), T7-

SF2/ASF-Mut.RS (lane 4), T7-SF2/ASF-RRM1 (lane 5), and T7-SF2/ASF-Mut.RRM1 (lane 6) expression plasmids. E. Subcellular localization of SF2/ASF and its truncated forms in PHFA cells.

#### **4.2.6 Suppression of SF2/ASF promotes JCV replication in astrocytes.**

Examination of SF2/ASF levels in primary fetal and adult astrocytes showed lower levels of this protein in fetal astrocytes compared to those in adult astrocytes, which had more than a three-fold higher level (Fig. 4.8A). This was an interesting observation as earlier results from our lab showed poor infection by JCV of adult astrocytes compared to fetal astrocytes. To investigate the involvement of SF2/ASF on JCV replication in these cells, we down-regulated expression of SF2/ASF with a lentivirus encoding shRNA that specifically targets SF2/ASF expression and demonstrated that the introduction of lenti-SF2/ASF shRNA but not the control lentivirus in JCV-infected cells increased viral production in both fetal and adult astrocytes (Fig. 4.8B). Results from Western blot analysis verified an increased level of viral capsid protein production, VP1, in cells with a decreased level of SF2/ASF (Fig. 4.8C). Further, results from immunocytochemical staining of the infected cells showed 53% and 136% increase in the number of VP1-positive cells in fetal and adult infected astrocytes, respectively. Of note, lenti-SF2/ASF shRNA was able to suppress 64% and 72% of SF2/ASF expression in adult and fetal astrocytes, respectively (shown in Fig. 4.8C). Thus, it is evident that a modest decrease in the level of SF2/ASF can augment the level of JCV replication in permissive cells.



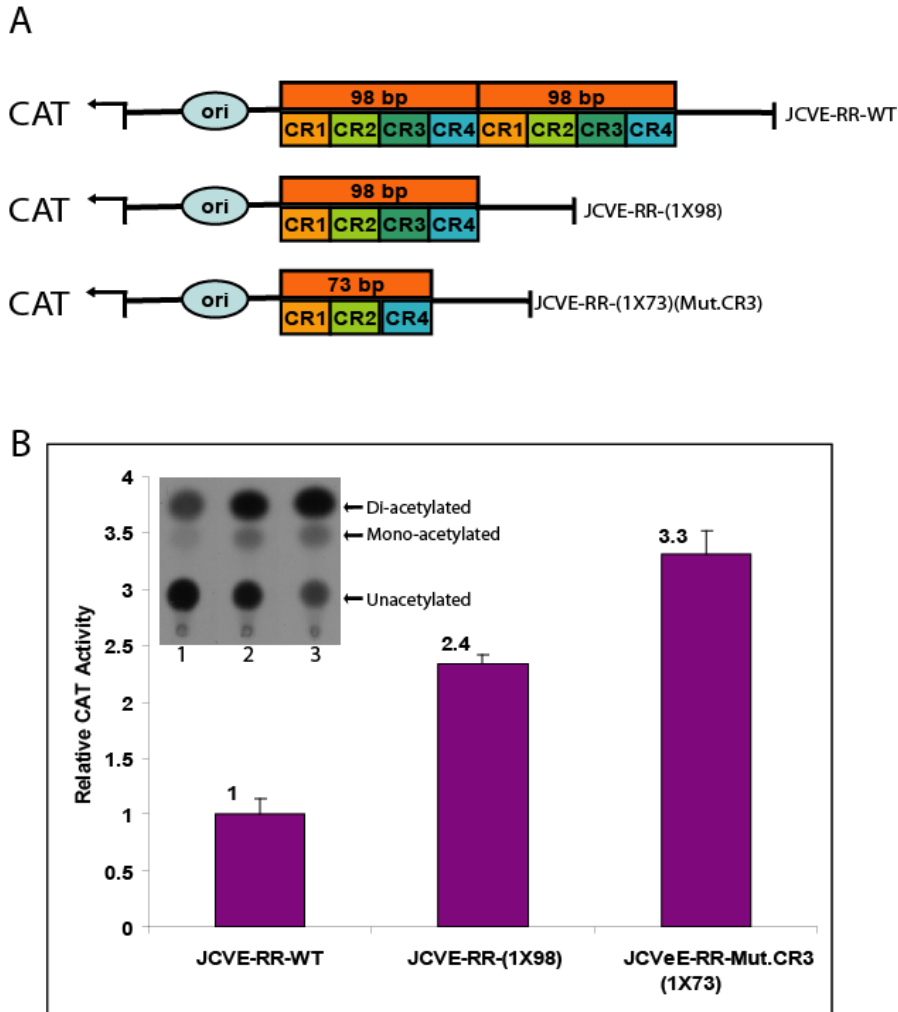
**Figure 4.8: Downregulation of SF2/ASF induces JCV propagation in primary human fetal and adult astrocytes.** A. Western blot analyses of SF2/ASF expression in PHFA and PHAA cells. Grb2 was probed as a loading control.

Band intensities were quantified and shown as a bar graph. B. PHFA and PHAA cells were infected with a lentivirus encoding SF2/ASF-shRNAs at day 0, and were transfected/infected with the JCV Mad-1 strain at day 1. Q-PCR analyses of JCV viral particles in culture supernatants were performed as described in Fig. 4.1C. C. Whole cells extracts were prepared from the same infections as described in panel B and processed for Western blotting by using specific antibodies against SF2/ASF, T-Ag, and VP1. Grb2 was probed as a loading control. D. Immunocytochemical analyses of VP1 expression in PHFA and PHAA cells infected with JCV. E. Quantification of VP1 expression in infected cells from panel D.

#### **4.2.7 Negative regulation of viral transcription is mediated by CR3 region of viral promoter.**

SF2-mediated suppression of JCV transcription was mainly dependent on the physical interaction of the protein with the JCV regulatory region mapped to the “CR3” locus of the promoter (Fig. 4.7). The Mad1 strain of JCV consists of two 98 bp repeated regions in its promoter, and contains two binding sites for SF2/ASF. We first created a reporter construct which lacked the second 98 bp repeated region and contained only one binding site for SF2/ASF. In the third construct, the CR3 region was also mutated by deletion (Fig. 4.9A). PHFA cells were transiently transfected with these constructs and the transcriptional activity was determined by CAT assay as described in the materials and methods. As seen in Fig. 4.9B, the reporter construct carries only one binding site for

SF2/ASF and showed one fold higher activity than the WT construct. Moreover, the third reporter construct with no binding site for SF2/ASF (JCV-RR-Mut.CR3-(1x73)) showed significantly more activity than JCV-RR-(1X98) and JCV-RR-WT, respectively.

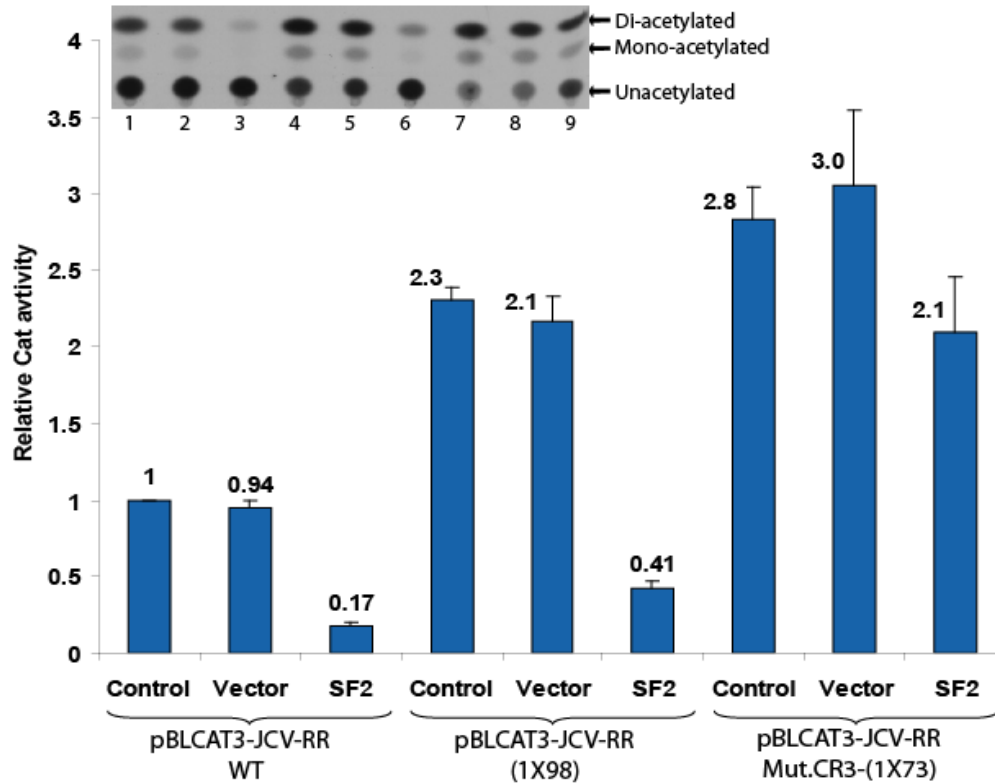


**Figure 4.9: Mutational analyses of JCV promoter.** A. Schematic representation of pBLCAT3 constructs that contained the JCV promoter sequences. Deletion mutations were created based on the Mad1 strain of JCV.

B. CAT enzyme activity of JCV promoter constructs were detected, and presented as a bar graph. The autoradiograph represents one of the three independent experiments performed.

#### **4.2.8 Effect of SF2/ASF on transcription mediated by JCV-(1X98) and JCV-Mut.CR3-(1X73).**

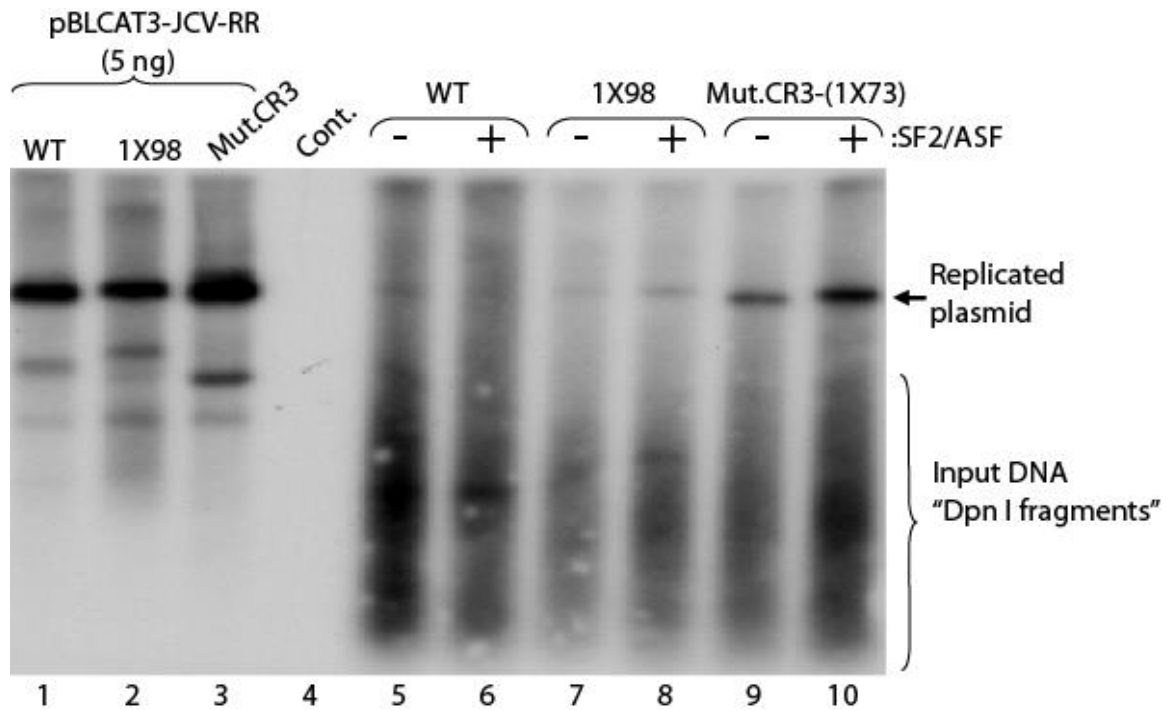
Mutational analyses of the JCV promoter region suggested that the viral promoter with one 98 bp repeated region and the one with CR3 deletion showed significantly higher transcriptional activity than the wild type promoter (Fig. 4.9). Next, we analyzed the effect of SF2 on the transcriptional activity of these mutant JCV-promoter sequences by CAT assay. As expected, SF2/ASF suppressed transcription induced by the wt JCV promoter and by the mutant promoter, which carries only one 98 bp repeated region (Fig. 4.10, compare control, vector, and SF2 bars). As hypothesized, SF2/ASF did not show any significant suppression on transcription mediated by the JCV-Mut.CR3-(1X73) promoter, which lacks both “CR3” regions (Fig. 4.10). These results suggest that binding of SF2/ASF to the JCV promoter through the CR3 region might be crucial for the suppression of viral transcription.



**Figure 4.10: Effect of SF2/ASF on transcription induced by mutant JCV promoter sequences.** pBLCAT3-JCV-RR WT, pBLCAT3-JCV-RR-(1X98), and pBLCAT3-JCV-RR-Mut.CR3-(1X78) reporter plasmids were transiently transfected into PHFA cells in the presence or absence of pCGT7 vector or pCGT7-SF2/ASF expression vector. CAT enzymatic activities were detected and calculated as described in the materials and methods.

#### **4.2.9 JCV-Mut.CR3-(1X73) replicates more efficiently than JCV-WT and JCV-(1X98).**

In order to test the effect of mutations of the JCV promoter on replication, we utilized the pBLCAT3-JCV-WT, pBLCAT3-JCV-(1X98), and pBLCAT3-JCV-Mut.CR3-(1X73) constructs described in Fig. 4.9. U87 MG cells were transfected with these constructs, and low molecular weight plasmid DNA was isolated at 72 hours post-transfection. Southern blot analyses of newly replicated plasmid DNAs were performed as described in the materials and methods. As expected, JCV-WT replicated in U87 MG cells with a low efficiency (Fig. 4.11, lane 5). CAT plasmid with one 98bp repeated region showed very similar replication efficiency as WT plasmid (compare lanes 5 and 7). Surprisingly pBLCAT3-JCV-Mut.CR3-(1x73) plasmid, which lacks the SF2/ASF binding site, replicated much more efficiently than pBLCAT3-JCV-WT and pBLCAT3-JCV-(1X98) constructs (compare lane 9 with lanes 5 and 7). We also analyzed the possible impact of SF2 on replication of JCV. As expected, SF2/ASF did not affect the replication of JCV mediated by wild type and by mutant with one 98bp repeat (Fig. 1.11). Interestingly, SF2 slightly induced the replication of the pBLCAT3-JCV-Mut.CR3-(1X73) construct (compare lanes 9 and 10). The actual mechanism of the increased replication of this construct mediated by SF2/ASF is not known.

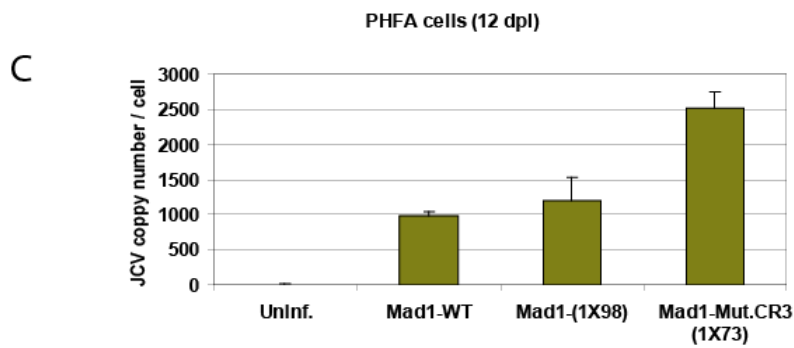
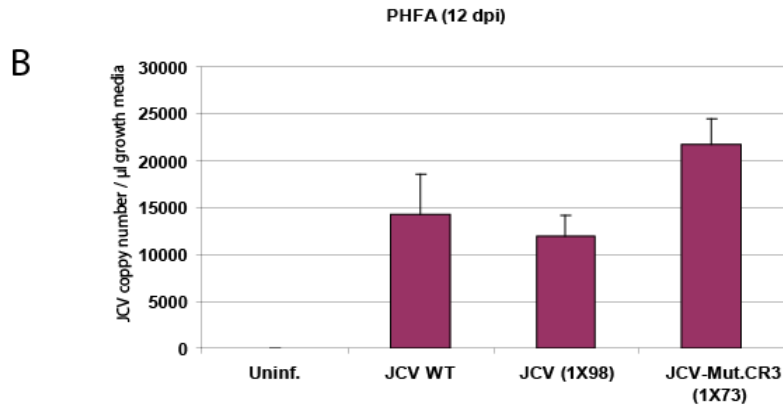
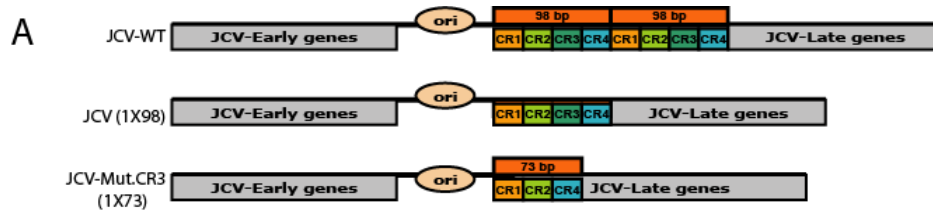


**Figure 4.11: Effect of SF2/ASF on replication of JCV.** Southern blot analyses of replicated pBLCAT3 plasmid DNA induced by mutant JCV promoter sequences. Probe was PCR-amplified CAT gene from the backbone of pBLCAT3 plasmid.

**4.2.10 JCV-Mad1.Mut.CR3-(1X73) virus propagates and replicates more efficiently than JCV-Mad1-WT and JCV-Mad1-(1X98) viruses in primary human fetal astrocytes.**

Reporter gene analyses of mutant JCV promoter sequences (Fig. 4.9 and 4.10) suggested that CR3 region of JCV TCR limits the transcription of the virus. However, these experiments were performed in the absence of viral early and late genes, which might also influence the activity of viral transcription induced by

wild type and mutant JCVs. In order to test the possible impact of the “CR3” region of JCV TCR on viral propagation, the same promoter mutations in these reporter constructs (Fig. 4.10, panel A) were created on JCV background (Fig. 4.12, panel A). PHFA cells were transfected/infected with these constructs. Released JCV particles in growth medium of infected cells were analyzed and quantified by Q-PCR analyses as described in the material and methods. As shown in figure 3.12, panel B, JCV-Mad1.Mut.CR3-(1X73) showed significantly higher levels of viral copies in growth media of cells. The replication rate of wild type JCV and JCV with mutations were also analyzed in infected PHFA cells. Consistent with Q-PCR analyses of viral copies in the growth media, Q-PCR analyzes of replicated viral genomes in infected PHFA cells also showed an increased replication rate of JCV-Mad1.Mut.CR3-(1X73) than either JCV-Mad1-WT or JCV-Mad1-(1X98) (Fig.4.12C).



**Figure 4.12: Propagation and replication of mutant JCVs in PHFA cells.** A. Genomic organization of JCV-Mad1-WT and mutants, JCV-Mad1-(1x98), and JCV-Mad1-Mut.CR3-(1X73) are illustrated. B. Q-PCR analyses of the JCV copy numbers in growth media of the infected PHFA cultures. C. Q-PCR analyses of the replicated viral DNA in the PHFA cells. Q-PCR analyses of JCV copy numbers in growth medium and in low molecular weight DNA extracted from the cells were described in the materials and methods.

### 4.3 DISCUSSION

It is believed that JCV may enter many cell types, yet only under certain immunosuppressive conditions does it enter into a lytic infection cycle in glial cells. Earlier studies established that cell type-specific reactivation of JCV in glial cells is primarily regulated at the transcriptional level (White et al., 2006, and 2009). In this respect, several transcription factors including YB-1, Pur $\alpha$ , GF-1, Egr-1, c-jun, NF1, and others were identified based on their ability to modulate JCV promoter activities in glial cells. Of note, none of these regulators were restricted to glial cells, and their overexpression, in most areas, failed to stimulate transcription of JCV in non-glial cells, suggesting that the JCV promoter in general is under negative regulation in non-permissive cells. Accordingly, one may speculate on the involvement of an inducible glial specific activator which becomes reactivated and promotes JCV gene transcription and replication during the course of immunosuppression. Thus, it is conceivable that the combination of ubiquitous negative and inducible positive glial specific factors determines the level of viral gene expression in glial and non-glial cells. Here, we identified an RNA splicing regulatory protein, SF2/ASF, which is ubiquitously expressed in all cell types as a negative regulator of JCV gene transcription. This is not a universal event for other members of the polyomavirus family, including SV40 and BKV, and requires a specific DNA sequence located within the 98 bp tandem promoter region. Our results demonstrate that a decrease in the level of SF2/ASF increases the level of viral gene expression and replication in glial cells and has no positive impact on JCV expression in non-glial cells, suggesting that

a positive glial-specific activator is required to initiate viral gene expression. SF2/ASF also suppresses expression of the JCV promoter in transformed cells whose viral genome is integrated in host chromosomes. Our DNA binding experiments identified a CR3 region of the JCV 98 bp repeat that is conserved in PML (Mad) and non-PML (archetype) strains of JCV as the primary target for SF2/ASF. This region encompasses several predicted binding sites for DNA binding proteins, including Pur $\alpha$ , NF-1, MF3, Elk-1, COE1, p300, and Zic3. At present, the importance of these transcriptional regulators and their potential interplay with SF2/ASF remains to be investigated.

SF2/ASF exhibits a modular structure consisting of two copies of RNA binding motifs (RRM1 and RRM2), followed by an arginine-serine rich (RS) domain (Zuo, and Manley, 1993). Whereas the RRM domains are responsible for the RNA binding activity of ASF/SF2, the RS region interacts with the core-splicing components (Sanford et al., 2005; Caceres et al., 1997). Our results showed that the RRM1 domain has the capacity to interact with the DNA sequence of JCV and that this interaction is important for suppression of viral gene transcription.

Examination of SF2/ASF levels in several glial-derived cells, including human glioblastoma, U-87 MG, primary human fetal astrocytes (PHFA) and primary human adult astrocytes (PHAA), showed higher levels of SF2/ASF expression in U-87 MG and PHAA where virus replicates poorly in comparison to PHFA, which is routinely utilized for virus propagation. Our results demonstrated that a decrease in the level of SF2/ASF in both PHFA and PHAA increases the

level of viral replication. In addition, deletion of the “CR3” region on JCV TCR increased the rate of transcription and propagation of the virus. Altogether, results from these studies identified a novel regulatory pathway involving the splicing factor SF2/ASF in the suppression of JCV gene transcription. This is the first report on the ability of SF2/ASF to control viral gene expression at the transcriptional level and suggests the operation of a novel regulatory event that controls reactivation of JCV in non-glial as well as glial cells.

## CHAPTER 5

### EXTINCTION OF TUMOR ANTIGEN EXPRESSION BY SF2/ASF IN JCV- TRANSFORMED CELL LINES

#### 5.1 INTRODUCTION

JCV is a human polyomavirus, whose activation mostly under immunosuppressive conditions in latently infected individuals leads to the development of progressive multifocal leukoencephalopathy (PML). In addition to JCV's primary role in the development of PML, JCV has also been shown to be associated with various tumors in laboratory animals and humans. JCV can transform primary human fetal glial cells in a similar manner as simian polyomavirus, SV40 (Mandl et al., 1987; Major et al., 1985). JCV-transformed primary human cells express viral-early genes and exhibit a transformed phenotype (Gallia et al., 1998). Inoculation of JCV in owl and squirrel monkeys induces glioblastomas, neuroblastomas, and astrocytomas (London et al., 1978 and 1983). Transgenic animals expressing the JCV-early genome result in the development of mesenteric tumors (Franks et al., 1996) and pituitary adenomas (Gordon et al., 2000). The oncogenic potential of JCV is strongly related to the expression of viral large and small tumor antigens. Ample amount of evidence suggests that the mechanism of JCV-mediated transformation relies on the suppression of tumor suppressor proteins, p53, pRb, and p130, by the viral large T antigen. Binding of large T antigen appears to interfere with the cell cycle properties of these proteins. Immunohistological studies of various tumors

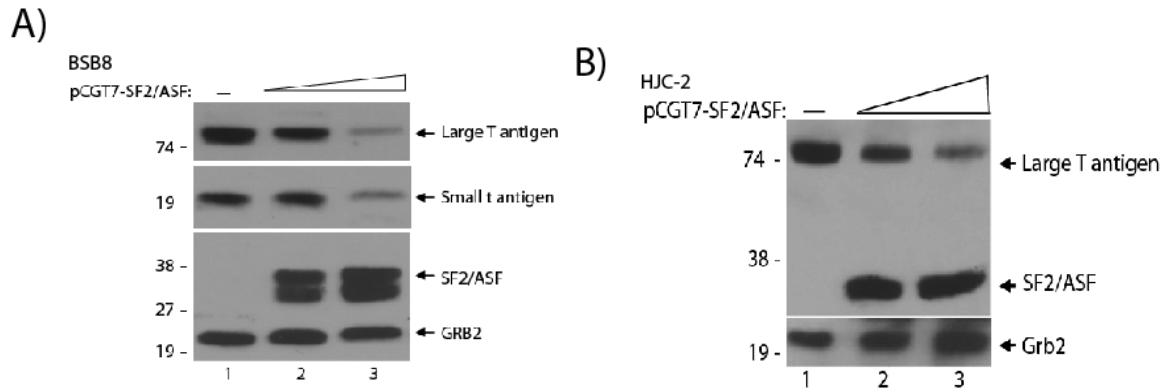
induced in JCV-transgenic animals revealed that only a small population of tumor cells exhibit viral large T antigen expression (Krynska et al., 2000). More interestingly, inoculation of the T-antigen-positive but not the T-antigen-negative cell lines obtained from JCV-induced medulloblastomas resulted in the development of massive tumors in experimental animals (Krynska et al., 2000).

We have recently demonstrated that the cellular alternative splicing factor, SF2/ASF, strongly regulates JCV early and late gene expression by directly targeting a double-stranded DNA motif within the viral promoter (Sariyer and Khalili, 2010). Here we investigated the possible impact of SF2/ASF on JCV-mediated transformation. Expression of SF2/ASF in JCV-induced cell lines strongly suppressed the expression of the large T antigen, and caused growth arrest, and induced apoptosis. On the contrary, down regulation of SF2/ASF in such tumor cell lines increased the growth rate of the cells in soft agar. These observations may suggest a novel role of SF2/ASF in JCV-mediated cellular transformation and provide a new avenue of research to better understand the mechanism of JCV-induced tumors.

## 5.2 RESULTS

### 5.2.1 JCV-early gene expression is suppressed by SF2/ASF in JCV-transformed cell lines.

The possible impact of SF2 on JCV tumor antigen expression in the viral transformed BsB8 and HJC-2 cell lines was tested by transient transfection studies. BsB8 cells were transfected with an SF2 expression plasmid, and whole-cell extracts were prepared at 48 hours post-transfection. Expression of tumor antigens (large T antigen and small t antigen) was detected by western blotting. As shown in Fig. 5.1, expression of SF2/ASF suppressed large T antigen and small t antigen expression in BsB8 cells, a cell line originated from a medulloblastoma developed in JCV-early transgenic mice. The effect of SF2 on tumor antigen expression was also tested in HJC-2 cells, a cell line obtained from glioblastoma induced by intracranial injection of JCV in hamsters. Consistent with BsB8 cells, expression of SF2 greatly suppressed large T antigen expression in these cell lines (compare lanes 2 and 3 with lane 1). Interestingly, western blot analyses of SF2/ASF expression in BsB8 cells revealed a double band formation. However, a single band corresponding to the right size of SF2 was observed in HJC-2 cells. The observed difference of SF2/ASF expression between both cell lines is not known.



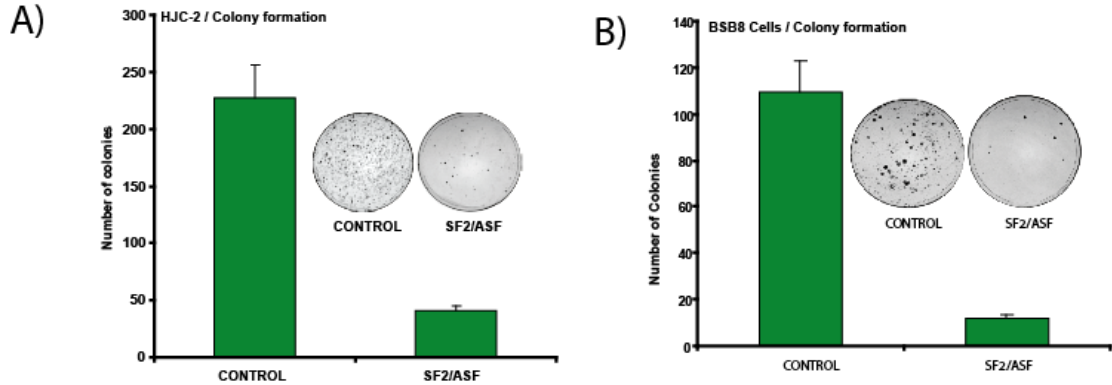
**Figure 5.1: SF2/ASF inhibits tumor antigen expression in JCV-transformed cell lines.** A. Western blot analyses of JCV tumor antigen expression in BSB8 cells. Cells were transfected with increasing concentrations of an SF2 expression plasmid. Total DNA amount per transfection was adjusted with empty vector. Expressions of large tumor antigen, small tumor antigen, and SF2/ASF were detected western blot using specific antibodies at 48 hours post-transfection. Grb2 was probed as loading control. B. Western blot analyses of tumor antigen expression in HJC-2 cells overexpressing SF2/ASF were performed as described in panel A.

### 5.2.2 SF2/ASF expression inhibits the transformed phenotype of JCV-induced tumor cells.

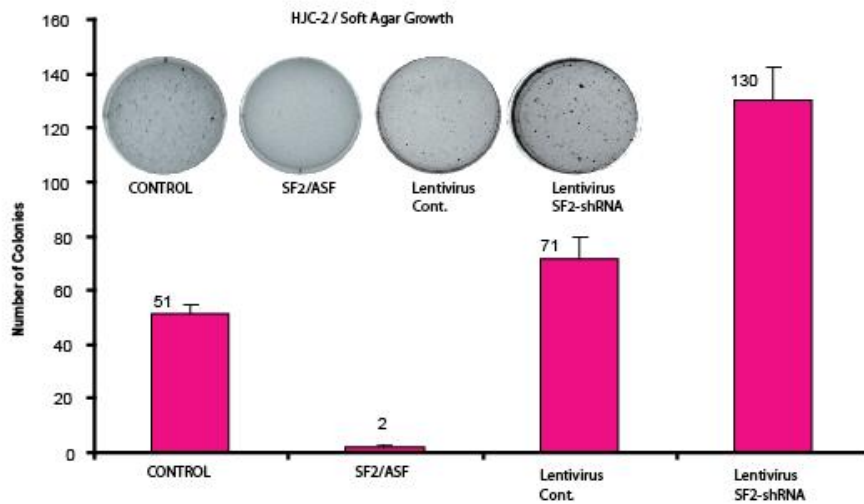
Previous studies suggested that the transformed phenotype of BSB8 cells is highly related to the maintained expression of viral tumor antigens. Subcutaneous inoculation of large T antigen-positive but not-negative tumor cell lines obtained from JCV-early gene transgenic mice resulted in formation of tumors in nude mice. As shown in Fig. 5.1, overexpression of SF2/ASF caused

an extinction of large T antigen expression in BsB8 and HJC-2 cells. Next, we tested the possible effect of SF2 on the transformed phenotype by colony formation assay. BsB8 and HJC-2 cells were transiently transfected with pCGT7-SF2 expression vector and pcDNA 3.1 zeu (+) which carries the G418 resistance gene. As seen in Fig. 5.2, BsB8 and HJC-2 cells formed colonies upon treatment with G418. However, expression of SF2/ASF greatly suppressed the number of colonies in both cell lines when compared to control groups (Fig. 5.2A and B, compare column 1 with column 2).

Next, we tested the effect of SF2/ASF on the growth of HJC-2 cells under anchorage-independent conditions. HJC-2 cells were either transfected with pCGT7-SF2/ASF expression plasmid or infected with a lentivirus encoding the shRNAs against SF2/ASF. The same number of cells from both conditions was plated on soft agar. Colony formation in soft agar was observed around the second week of after plating cells, and cells were fixed and stained at the 3<sup>rd</sup> week after plating. As it was expected, HJC-2 cells formed nice colonies on soft agar under anchorage-independent conditions (Fig. 5.3, first column). Results from three independent experiments revealed that expression of SF2/ASF significantly inhibited colony formation on soft agar (Fig. 5.3). HJC-2 cells infected with lentiviruses expressing shRNA sequences against SF2/ASF formed significantly more colonies than the control cells and those infected with lentivirus alone.



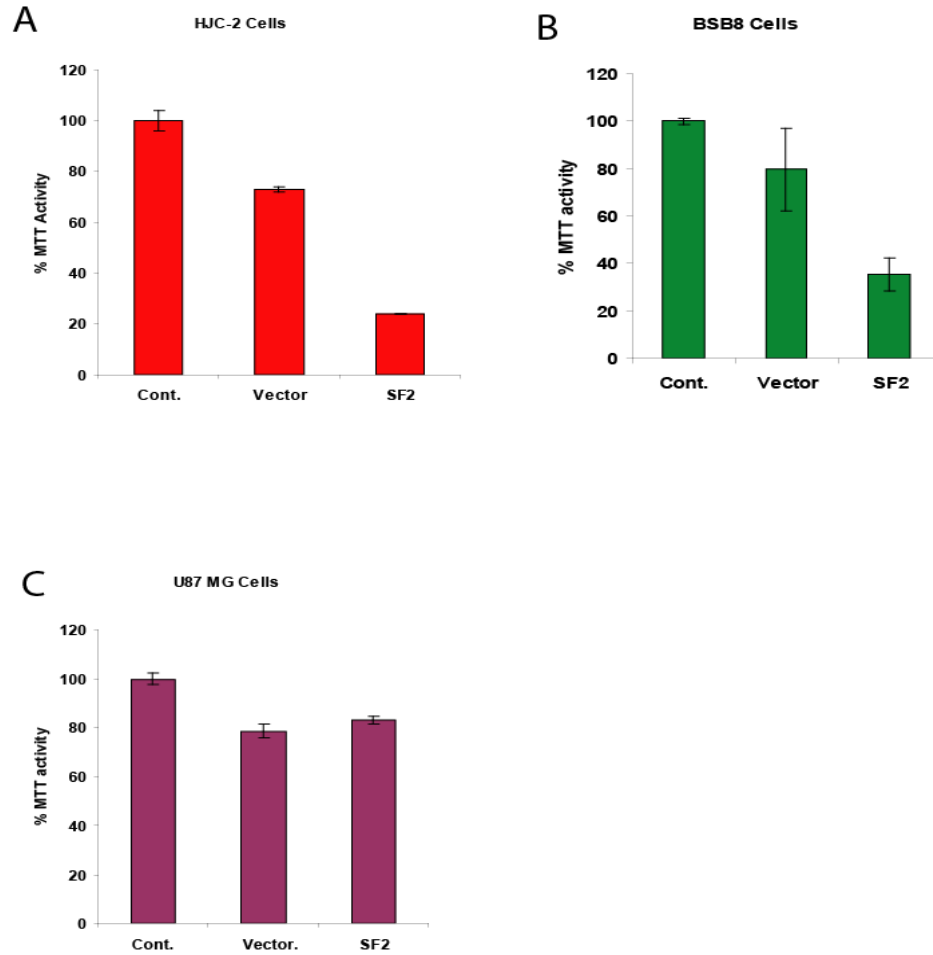
**Figure 5.2: Colony formation of HJC-2 and BsB8 cells transfected with the SF2/ASF expression plasmid.** HJC-2 (panel A) and BsB8 (panel B) cells were seeded in 100 mm plates and transfected with 10  $\mu$ g of either empty plasmid or SF2/ASF expression plasmid. Twenty-four hours after transfection, cultures were harvested and equal numbers of cells (100,000) were placed in 100 mm plates containing DMEM plus fetal calf serum (10%) and 1mM G418. After incubation for two weeks, cells were washed with PBS and fixed with solution containing 1% methylene blue for 10 minutes. All experiments were carried out in triplicate and the figure depicts representative data.



**Figure 5.3: SF2/ASF inhibits the anchorage-independent growth of HJC-2 cells in soft agar.** Approximately  $5 \times 10^5$  HJC-2 cells were transfected with pCDNA 3.1 plus pCGT7 (control) and pCDNA 3.1 plus pCGT7-SF2/ASF (SF2/ASF) constructs. Twenty-four hours after transfection, cells were trypsinized, and counted, and 10,000 cells were seeded into soft agar media containing 1mM G418 as described in the materials and methods. In the second round of soft agar experiments, HJC-2 cells were plated ( $5 \times 10^5$ ) in 60mm dishes, infected with either control lentivirus (Lentivirus cont.) or with lentivirus containing an shRNA sequence against SF2/ASF (lentivirus SF2-shRNA). Forty-eight hour after infection, cells were trypsinized, counted, and 10,000 cells were seeded into soft agar media. Plates were incubated at  $37^\circ\text{C}$  for three weeks, and colonies were counted. Each experiment was performed in triplicate.

### **5.2.3 SF2/ASF inhibits proliferation of the JCV-transformed cells.**

The colony formation and growth on soft agar experiments suggested that SF2/ASF suppresses colony formation and inhibits growth of JCV-transformed cells on soft agar. In the next series of experiments, we analyzed the effect of SF2/ASF on cellular viability of HJC-2, BsB8, and U87 MG cells by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Cells were plated in six-well plates ( $2 \times 10^5$ ) and transfected either with pCGT7 empty vector or with pCGT7-SF2/ASF expression plasmid. MTT activities were detected as described in the materials and methods. As shown in Fig. 5.4, ectopic expression of SF2/ASF greatly suppressed the MTT activities of HJC-2 and BsB8 cells (Fig. 5.4A-B). MTT activity of U87 MG cells ectopically expressing SF2/ASF was also analyzed. Unlike those JCV-transformed cells, HJC-2 and BSB8, the MTT activity of U87 MG cells was not effected by SF2/ASF (Fig. 5.4C), suggested that SF2/ASF specifically inhibited the growth of JCV-transformed cells.



**Figure 5.4: Inhibition of Bsb8 and HJC-2 cells viability by SF2/ASF.** HJC-2 (A), Bsb8 (B), and U87 MG (C) cells were transfected either with empty vector or with an SF2 expression plasmid. Seventy-two hours after transfection, MTT activities were detected. MTT activity of untransfected cells was accepted as 100%, and the MTT activity of study groups was calculated accordingly.

### 5.3 DISCUSSION

The human polyomavirus, JCV, has been shown to be associated with various tumors in laboratory animals and humans. JCV can transform primary human fetal glial cells in a similar manner as the simian polyomavirus, SV40 (Mandl et al., 1987; Major et al., 1985). Transgenic animals expressing the JCV-early genome often develop CNS-origin tumors (Franks et al., 1996, Gordon et al., 2000). Interestingly, immunohistological studies of various tumors induced in JCV-transgenic animals revealed that only a small population of tumor cells express viral Large Tumor antigen, LT-ag. More interestingly, inoculation of the T-antigen-positive but not the T-antigen-negative cell lines obtained from JCV-induced medulloblastomas resulted in the development of massive tumors in experimental animals (Krynska et al., 2000). Considering the fact that all of the cells in these transgenic animals retain the JCV-early genome, the mechanism for the extinction of T-antigen expression in tumor tissue is unknown.

Here, we investigated the possible impact of SF2/ASF on the transformed phenotype of JCV-induced tumor cell lines. SF2/ASF is a cellular alternative splicing factor mainly involved in the post-transcriptional regulation of gene expression by forming a complex with a spliceosome. However, we have previously shown that SF2/ASF targets a specific DNA motif (CR3 region) within the JCV bidirectional promoter and strongly inhibits transcription of viral early and late genes. SF2/ASF-mediated inhibition of viral genes at the transcriptional level is specific to JCV and requires the viral promoter. These experiments were mainly done by transient transfection studies, and viral genes were ectopically

expressed. In order to investigate impact of SF2 on the expression of viral genes, we utilized HJC-2 and BsB8 cells, which contain an integrated viral genome and express LT-ag and smt-ag under the control of viral promoter. Western blot analyzes of HJC-2 and BsB8 cells have revealed that overexpression of SF2/ASF caused inhibition of tumor antigen expression in both cell lines, suggesting that SF2/ASF can also inhibit expression of JCV genes driven by integrated copies of the viral genome into the host chromosomes. Analyses of the transformed characteristics of these cell lines by colony formation and soft agar assays revealed that SF2/ASF suppresses proliferation and growth of the cells. In other words, SF2-mediated extinction of tumor antigen expression resulted in loss of transformed characteristics of these cell lines. Moreover, SF2/ASF expression decreased viability and induced apoptosis in JCV-induced tumor cell lines, but not in a well known human glioblastoma cell line, U87 MG.

These observations provide further evidence that SF2/ASF is a critical cellular protein which limits and controls expression of JCV genes and may suggest a novel role of SF2/ASF in JCV-mediated cellular transformation, thus providing a new avenue of research to better understand the mechanism of JCV-induced tumorigenesis.

## CHAPTER 6

### CONCLUSIONS AND FUTURE DIRECTIONS

#### 6.1 New Hybrid Cell Lines That Support JCV Propagation.

Viruses utilize cellular replication and transcription machinery in order to propagate and cause disease in susceptible hosts. Viral infection starts with the cellular receptor available for the virus to enter cells. The second step involves the successful transcription of viral immediate-regulatory genes that will put the cell in a suitable state in order for the virus to start replicating its genome and to transcribe the viral structural proteins. Finally, the third stage of infection is the assembly and release of mature viral particles to continue the viral replication cycle. Previous studies on JCV replication and transcription indicate that tissue-specific gene expression might be the main determinant in JCV tropism (Khalili et al., 2005; Major et al., 1992; Safak and Khalili, 2003; Safak, Major, and Khalili, 2005). The absence of a suitable JCV animal model leaves the tissue culture system as the only available tool for the study of the molecular mechanisms involved in the JCV infection cycle. Current cell culture systems for JCV are limited to primary cultures of astrocytes and transformed cell lines of glial origin, and therefore, there is a need for establishing a cost-effective and suitable cell line for the study of the biology of JCV.

As an alternative to primary cultures of human fetal astrocytes, we have produced hybrid human cell lines by fusing primary human fetal astrocytes with human malignant glioblastoma cells (U-87MG) deficient in the HPRT enzyme.

We identified many JCV-permissive hybrid cell lines and examined two of these clones with respect to hybrid properties and viral propagation. We infected and compared hybrid cell lines with PHFA cells for viral transcription and replication. We demonstrated that the newly-generated hybrid cell lines support JCV replication at early passages but lose that property at late passages.

One of the main drawbacks of hybridoma cell technology is the instability of the genome of the hybrid clones (Ganem, Storchova, and Pellman, 2007). We think that this gradual loss of JCV replication during late passages of the hybrid lines may reflect genomic instability of our clones. Another possibility for restricted JCV replication in late passages is that it might be due to the activation of cell-type specific inhibitors of viral gene expression and replication. This possibility is consistent with earlier fusion studies, in which mouse fibroblasts fused with transformed hamster glial cells lost expression of JCV large T antigen (Beggs, Frisque, and Scangos, 1988; Beggs, Miner, and Scangos, 1990). This observation indicated the induction of new cellular factors that negatively regulate JCV gene expression in the hybrid cells. Further analyses of the gene expression profiles of the newly-generated hybrid clones may lead to the identification of novel cellular factors which may specifically regulate JCV gene expression in glial cells.

In conclusion, we have established several hybrid cell lines using human primary fetal astrocytes and human malignant glioblastoma (U-87MG) deficient in the HPRT enzyme. We have chosen two hybrid lines, HC-7 and HC-15, for further analysis and demonstrated that both hybrid lines support JCV replication

and propagation at early passages but lose that property at late passages. Nevertheless, these newly-generated hybrid cell lines may serve as a good tissue culture model system to identify cellular determinants of JCV replication and propagation and may help us to develop more effective strategies to prevent JCV infection in glial cells.

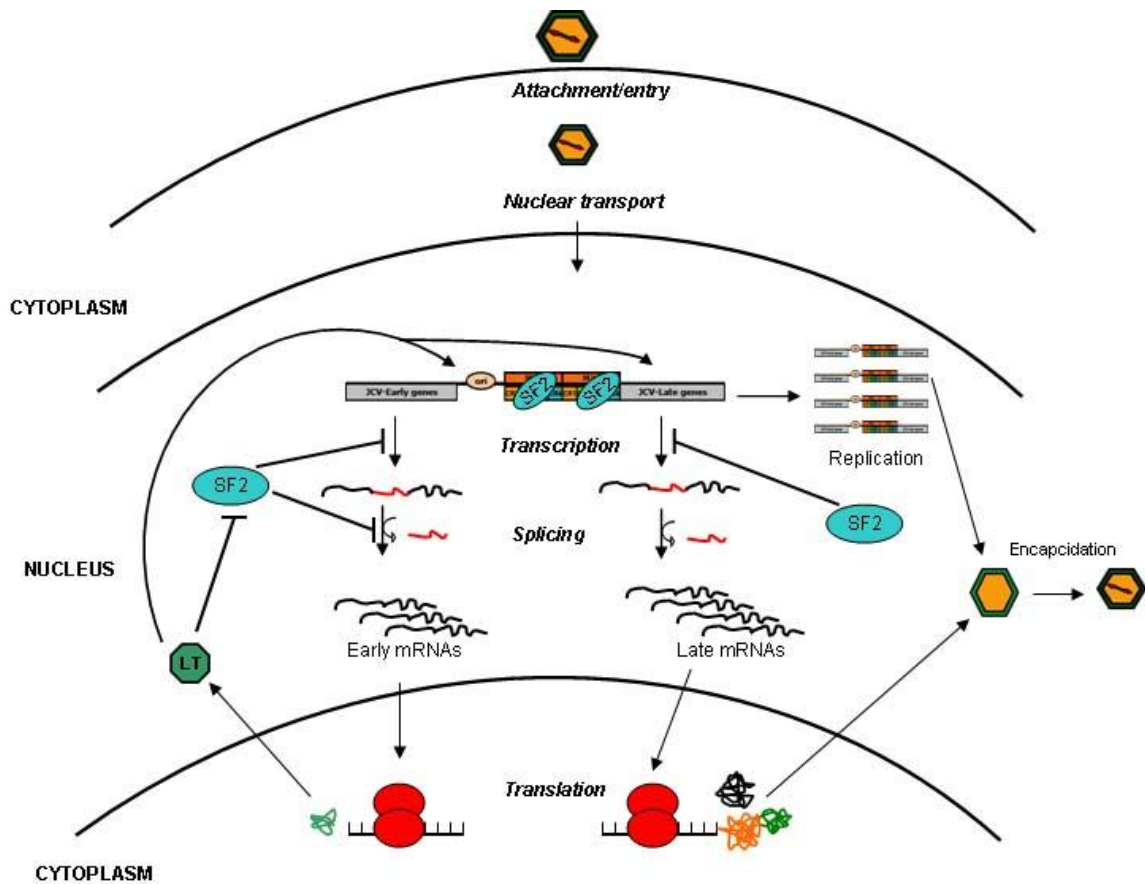
## **6.2 Identification and Characterization of SF2/ASF as a Negative Regulator of JCV Gene Expression.**

JC virus infection commonly occurs in early childhood and establishes a latent infection in healthy individuals for the rest of their life. Under immunosuppressive conditions, such as AIDS and lymphoproliferative diseases, the virus reactivates from latently-infected tissues, is transmitted through the blood (possibly by B-cells), and reaches the brain, where it preferentially infects and replicates in glial cells. Although JCV can enter/infect almost all types of different organs and tissues, it can only complete its life cycle in the CNS. The mechanism of JCV tropism in the CNS, specifically in glial cells, has not yet been clarified. Most research has been focused on the identification of glial-specific transcription factors. As an outcome of these studies, many transcription factors have been identified, such as p53, YB-1, NF-1, GF-1, SP1, and c-jun (White and Khalili, 2006). However, none of these transcription factors were either glial-specific or able to explain viral tropism. Another interesting feature of JCV is the slow progression of the replication cycle in primary fetal glial cultures, PHFG, the only primary cell culture system to grow virus in vitro. Upon infection

of PHFG cells, JCV early protein expression can be detected after several days, and the virus completes its replication cycle in several weeks. On the other hand, expression of viral early proteins of simian polyomavirus, SV40, starts around 8-10 h post-infection, and completion of the viral life cycle occurs in as early as 48 hours in CV-1 cells. Absence of glial-specific /JCV-specific transcription factors and slow progression of JCV even in the glial systems suggest that JCV gene expression might also be controlled by other cellular factors which suppress or slow viral propagation.

We show here that SF2/ASF limits the replication and progression of JC virus in glial cells. Overexpression of SF2/ASF during JCV infection resulted in a great reduction of viral replication and gene expression. SF2/ASF is a well characterized alternative splicing factor. Therefore, we hypothesized that the observed suppression of JCV replication could be due to a posttranscriptional block to the viral primary transcripts. We developed an RT-PCR-based approach to address this question in vivo. JCV early genes were expressed under the control of either JCV's own promoter or a CMV promoter. As hypothesized, SF2/ASF suppressed the splicing and caused an accumulation of the viral early pre-mRNA when expressed from the pCDNA3.1 expression plasmid. Surprisingly, SF2/ASF not only suppressed the splicing but also broadly inhibited the formation of the viral primary transcripts when expressed under the control of JCV's own promoter. In order to further characterize the SF2-mediated suppression of JCV transcription, we utilized a CAT-based reporter gene assay. Consistent with the observations from splicing experiments, SF2/ASF showed a

strong inhibition of not only JCV-early but also -late promoter activities. Furthermore, SF2/ASF did not show any inhibition on CAT activities driven by the E2F-1 promoter or the BKV early and late promoters.



**Figure 6.1: Proposed mechanism of SF2/ASF-mediated inhibition of JCV replication in glial cells.**

Specific inhibition of JCV transcription but not SV40 or BKV by SF2/ASF suggested that it could be directly involved in regulation of JCV transcription. We

showed that SF2/ASF was able to interact with the JCV regulatory region (JCV-RR) assessed by a ChIP assay *in vivo*. In order to test the direct interaction between SF2/ASF and JCV-RR, we incubated radiolabeled oligonucleotides spanning the 98 bp repeated region of JCV Mad-1 strain with recombinant SF2/ASF (GST-SF2). Interestingly, SF2/ASF specifically interacted with only the CR3 region of the viral promoter. We further characterized the interaction of SF2/ASF with CR3 oligonucleotide by gel shift assays and showed that SF2/ASF formed a complex with this unique region of JCV promoter. Removal of RNA from nuclear protein extracts before the binding studies greatly improved the binding efficiency of SF2/ASF to the CR3 oligonucleotide. Even though the DNA binding activity of an RNA binding protein is a rare event, SF2/ASF has been recently shown to interact with a dsDNA motif within a human rDNA replication origin (He et al., 2009). Our DNA binding studies, by using either nuclear extracts or recombinant proteins as a source of SF2/ASF, suggested that it also directly binds to a motif within the JCV promoter region, which represents the second report of the DNA-binding activity of SF2/ASF. While the exact mechanism responsible for the inhibition of JCV transcription by SF2/ASF remains to be elucidated, it is likely to involve a dose-dependent competition with some other transcription factors which are required for transcriptional activation of the virus. On the other hand, interaction of SF2/ASF with viral promoter might also interfere with the initiation and/or elongation of the transcription complex. Further studies of the action of SF2/ASF on the transcriptional regulation of JC virus through its

interaction with the viral promoter will provide more insight into this novel regulatory role of SF2/ASF.

SF2/ASF exhibits a modular structure consisting of two copies of RNA binding motifs (RRM1, RRM2), followed by an Arginine-Serine-rich (RS) domain (Zuo and Manley, 1993). While RRM1 and RRM2 domains determine the RNA-binding specificity, RS domain interacts with the core splicing components (Sanford et al., 2005; Caceres et al., 1997). In order to characterize the functional DNA binding module of SF2/ASF, we sequentially created truncated forms of the protein and performed a ChiP assay. We found that the RRM1 domain was mainly responsible for the interaction with JCV genomic DNA. We also tested the functional role of this interaction by CAT reporter assays. Consistent with the DNA binding studies, the RRM1 domain of SF2/ASF was also mainly responsible for the suppression of JCV transcription. These observations strongly suggest that direct interaction of SF2/ASF with the JCV regulatory region through the RRM1 domain is required for the suppression of viral transcription.

In addition to those gain-of-function studies, we also analyzed the impact of SF2/ASF on JCV propagation by loss-of-function studies. First, we compared the endogenous expression of SF2/ASF in primary human fetal (PHFA) and adult (PHAA) astrocytes, and U87 MG cells. Interestingly, SF2/ASF expression was ~2 and ~4 fold higher in PHAA cells than U87 MG and PHFA cells, respectively. PHFA cells are the only primary culture system which support viral multiplication and are used by us and others as a model system of JCV infection. On the other hand, JCV infection of PHAA cells has not yet been established. Here we also

showed that PHAA cells could also permit JCV infection with a ~ 6 to 10 fold lower efficiency than PHAA cells. Furthermore, downregulation of SF2/ASF expression resulted in a significant increase of JCV propagation in PHFA and PHAA cells, respectively. Further studies should address the functional importance of the differential expression of SF2/ASF in PHFA and PHAA cells and its role in the reactivation of JCV from the latency period and contribution to the pathology of the PML.

We also investigated the possible impact of SF2/ASF on the transformed phenotype of JCV-induced tumor cell lines. SF2/ASF caused an extinction of Large T antigen and small t antigen expression in the JCV-transformed tumor cell lines, BSB8 and HJC2. Analyses of transformed characteristics of these cell lines by colony formation and soft agar assays revealed that SF2/ASF suppresses the growth and proliferation of these cells. In addition, SF2/ASF expression decreased viability and induced apoptosis in these cell lines.

In conclusion, the data represented here have identified a novel regulatory pathway involving the splicing factor, SF2/ASF, in the suppression of JCV gene expression. These findings suggest a novel role of SF2/ASF in JCV-mediated cellular transformation and may provide a new avenue of research to better understand JCV-induced diseases.

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