CYTOKINE-MEDIATED REGULATION OF BK VIRUS REPLICATION

by

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ABSTRACT

BK virus (BKV) is a member of the polyomavirus family that infects nearly the entire human population at an early age. Following a subclinical primary infection, BKV is able to establish a persistent infection in the kidney and urinary tract. Reactivation of BKV occurs in immunocompromised individuals and can lead to severe disease. BKV-associated diseases include polyomavirus nephropathy (PVN), a form of acute interstitial nephritis that afflicts up to 10% of renal transplant recipients, and hemorrhagic cystitis (HC), an infection of the bladder characterized by inflammation and hematuria that affects 10% of bone marrow transplant recipients. PVN and HC are increasing in prevalence, likely due to the development of more potent immunosuppressive therapies.

To better understand which components of the immune system are important for regulating BKV infection, we began to examine the effect of cytokines on virus replication. Interferon-gamma (IFN-γ) has a strong inhibitory effect on BKV transcription, gene expression, and replication, but does not affect the kinetics of the viral life cycle or trafficking of the virus to the nucleus. IFN-γ treatment inhibited gene expression of three different BKV strains similarly, suggesting that regulation by this cytokine is relevant for all viral strains. We have begun to further examine regulation of viral transcription by IFN-γ to identify the specific factors involved and investigate changes in viral chromatin structure. In contrast to IFN-γ, TGF-β has an upregulatory

effect on BKV strain TU early promoter activity. Three other strains examined, however, were either unaffected or downregulated by TGF-β treatment in a cell type-dependent manner. The TGF-β response elements were mapped within the promoter of BKV TU. The viral strain- and cell type-dependent effects of TGF-β demonstrate the complex nature in which BKV is regulated by cytokine signaling.

Finally, we have begun to investigate the inability of archetype BKV strains to replicate in tissue culture. The linear structure of the non-coding control region (NCCR) distinguishes archetype from rearranged strains, which have duplications and deletions of NCCR sequences. Exchanging the NCCRs of archetype and rearranged genomes restores archetype DNA replication but prevents rearranged DNA replication. In addition, archetype DNA replication is observed in the presence of a TAg-expressing plasmid, suggesting that archetype viruses are limited in replication ability by their lower production of TAg. Overall, our findings will help us to better understand BKV persistence in healthy individuals and reactivation in immunocompromised patients.

CHAPTER I

INTRODUCTION

Viruses have long been studied for their ability to evade the host immune response and carry out their life cycles in the context of otherwise healthy individuals. For many viruses, this is primarily mediated by the production of viral gene products that mimic or block the mounting immune response. Encoding such proteins in the viral genome, however, requires genetic space and therefore small viruses are limited in their capacity to make these factors. BK virus (BKV) has a genome of only 5.2 kb and encodes only seven proteins, yet is able to establish a persistent infection in healthy individuals. This chapter will provide detailed information on the molecular biology, immunology, and clinical aspects of BKV to provide the necessary background for the discussion of viral regulation by the host immune response and establishment of persistent infection in the following chapters.

Genetics and Life Cycle of BK Virus

BKV is a member of the family *Polyomaviridae*, a group of viruses characterized by similar genome structures, small nonenveloped virions, and the ability to induce tumors in cells that do not support productive infections (Imperiale and Major, 2007). There are two known human polyomaviruses, BKV and JC virus (JCV), however recent reports of viral sequences isolated from respiratory samples and skin cancer cells suggest the existence of three additional human polyomaviruses, WU, KI, and Merkel cell polyomavirus (Allander et al., 2007; Feng et al., 2008; Gaynor et al., 2007). The polyomavirus family also includes the well-studied simian virus 40 (SV40) and mouse polyomavirus (Py); many details of BKV molecular biology are derived from what is known for these two viruses. BKV is highly homologous to JCV and SV40, sharing 78 to 90% homology at the amino acid level over the major viral proteins, and 75% and 69% DNA sequence homology throughout the genome, respectively (Cubitt, 2006; Imperiale, 2001). The polyomaviruses, however, are highly species specific and can only replicate efficiently in their natural host cells. In addition, there is evidence suggesting the coevolution of these viruses with their hosts (Shadan and Villarreal, 1993). Thus, SV40 and Py can only provide limited information about the immune response to BKV or the dynamics of infection, transmission, and persistence in humans.

BKV has a 40 to 45 nm icosahedral virion with a T = 7 lattice symmetry (Li et al., 2003). The virion is composed of 360 copies of VP1, arranged in 72 capsomeres, each of which contains five copies of VP1 and one copy of either VP2 or VP3 (Imperiale and Major, 2007). The genome is a circular, double-stranded DNA approximately 5 kb in length, and is associated with cellular histones H2A, H2B, H3 and H4 both in the nucleus

of infected cells and in the virion (Meneguzzi et al., 1978). This association with cellular histones leaves the virus vulnerable to regulation by host chromatin remodeling agents.

The genome of BKV has three major regions: the non-coding control region, the early coding region, and the late coding region (Figure 1.1). The non-coding control region (NCCR) contains a bidirectional promoter that drives the production of early region transcripts from one genomic strand and late region transcripts from the opposite strand. In addition, the NCCR contains the origin of replication, three TAg binding motifs, and many cellular transcription factor binding sites. The NCCR is the region of greatest variability between different isolates and thus distinguishes one “strain” of BKV from another (NCCR variants are henceforth termed strains in accordance with the literature). The NCCR is also the region of least conservation among members of the polyomavirus family. The early coding region contains the genes for large tumor antigen (TAg) and small tumor antigen (tAg), the transcripts of which are produced by alternative splicing of a common mRNA precursor. There is now evidence of a third early protein in BKV called truncated T antigen (truncTAg; D. Das, A. Joseph, J. Abend, D. Campbell-Cecen, and M. Imperiale, in preparation), that has a transcript structure similar to that of SV40 17kT and the T’ proteins of JCV (Trowbridge and Frisque, 1995; Zerrahn et al., 1993). The early region is highly conserved between the different BKV strains. The late coding region contains the genes for viral capsid proteins VP1, VP2, and VP3, and the agnoprotein, the transcripts of which are also all produced by alternative splicing of a common mRNA precursor. VP2 and VP3 are translated in the same reading frame using different start codons, such that VP3 is an N-terminal truncated form of VP2. VP1 is translated from the same transcript but using a start codon in a different reading frame

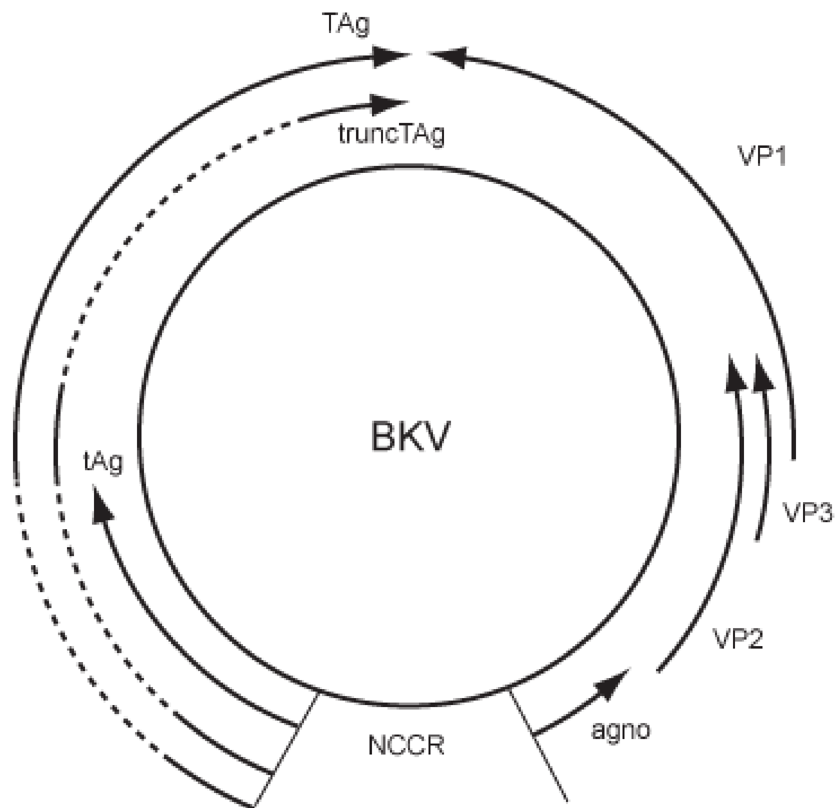

Figure 1.1. The genome of BKV. This schematic shows the circular, double-stranded DNA genome of BKV. The three major regions are shown as follows: the non-coding control region (NCCR, bottom section), the early coding region (left hemisphere), and the late coding region (right hemisphere). Solid arrows represent transcripts that encode the viral proteins; dashed lines represent the differential splicing of the early coding region. Figure courtesy of Mengxi Jiang.

than that of VP2 and VP3. The 5' end of the transcript has another open reading frame from which agnoprotein is translated. While the late proteins are highly conserved, there is a short region of variability (amino acids 61 to 83) within VP1 that is used to assign each strain of BKV to a particular subtype (I, II, III, IV; Jin, 1993; Jin et al., 1993). Subtype I viruses predominate in the human population, followed by subtype IV, while subtypes II and III are rare (Cubitt, 2006; Nukuzuma et al., 2006). The functional relevance of BKV subtypes is not yet fully understood.

The pathway for BKV entry and trafficking to the cell nucleus is not yet fully understood, but is somewhat distinct from that of JCV, SV40, and Py. The receptor for BKV is either of the gangliosides GD1b and GT1b (Low et al., 2006) or an N-linked glycoprotein with an $\alpha(2,3)$-linked sialic acid (Dugan et al., 2005); both reports note the importance of sialic acids in BKV receptor binding. Upon engaging the receptor, the virus is internalized in a caveolae-dependent manner, similar to SV40 but distinct from JCV, which enters by clathrin-dependent endocytosis, and Py, which enters via a unique endocytic pathway (Anderson et al., 1996; Atwood, 2001; Eash et al., 2004; Pho et al., 2000). The subsequent steps of BKV trafficking to the nucleus are currently under investigation, but the pathway includes passage of the virus through the endoplasmic reticulum and involves both microtubules and actin filaments (Eash and Atwood, 2005; Low et al., 2006; Moriyama and Sorokin, 2008). It is still unclear whether the partially disassembled virion or only the viral minichromosome is transported into the nucleus where replication occurs.

The first event to follow delivery of the viral genome to the nucleus is transcription of the early region and subsequent production of TAg and tAg. The

processes of transcription, translation, and DNA replication for BKV require the use of host cell enzymatic machinery, as the viral genome does not encode the proteins to accomplish these tasks. TAg is arguably the most important of the viral proteins as it assists and regulates the host cell machinery in these processes, performing many functions that are critical to the viral life cycle (based on findings for SV40; Cole and Conzen, 2001; Kierstad and Pipas, 1996; Kim et al., 2001; Moens and Rekvig, 2001). Once translated in the cytoplasm, TAg translocates back to the nucleus by means of a highly conserved nuclear localization signal at the N-terminus. TAg then interacts with the pRB family of proteins (pRB, p107, and p130), which releases the E2F transcription factor and drives the cell into S phase (Harris et al., 1996; Harris et al., 1998). There are three known binding sites for TAg at the origin of replication which mediate several functions: 1) inhibition of early gene transcription by blocking RNA polymerase binding, 2) initiation of viral genome replication by recruiting cellular proteins and acting as a helicase to facilitate the unwinding of the genome (Stahl et al., 1986; Stillman et al., 1985), and 3) activation and promotion of late gene transcription by binding to the late promoter (Deyerle et al., 1989; Salzman et al., 1986). TAg also binds and inactivates the tumor suppressor p53 to promote cell survival during infection (Harris et al., 1996; Shivakumar and Das, 1996). Overall, TAg establishes a cellular environment that is supportive of viral replication. Due to its importance to the viral life cycle, TAg expression may also be a key point of regulation by the host immune system.

Small T antigen is somewhat viewed as an auxiliary TAg and is distributed throughout the cytoplasm and nucleus (Cole and Conzen, 2001). It has been shown to enhance DNA replication (Cicala et al., 1994), mediate cell cycle progression (Howe et

al., 1998; Whalen et al., 1999), and aid in transformation of cells (Manfredi and Prives, 1994). A major function of tAg involves interaction with and inactivation of protein phosphatase 2A, which leads to cell proliferation and transformation; this is a unique function of tAg not shared by TAg (Skoczylas et al., 2004; Yang et al., 1991). It appears, however, that tAg is dispensable in the context of lytic infection, based on the ability of BKV strain MM to mediate a productive infection in the absence of detectable tAg expression (Seif et al., 1979).

The production of early proteins and the establishment of a suitable host cell environment promote replication of the viral genome. BKV DNA replication begins at the origin of replication, proceeds bidirectionally using host DNA polymerase α-primase to prime the extension by DNA polymerase δ, and ends at the opposite side of the genome, where DNA ligase I replaces missing nucleotides and topoisomerase II mediates the separation of the daughter molecules (based on SV40 replication; reviewed in Kim et al., 2001). Late gene transcription occurs concurrently and the capsid proteins (VP1, VP2, VP3) are translated and translocated back to the nucleus, the site of viral assembly. Lastly, agnoprotein is expressed at very late stages of lytic infection. BKV agnoprotein is localized to the cytoplasm and perinuclear region, and interacts with cellular proteins of various molecular weights (Rinaldo et al., 1998). Although the function is not well-defined, it has been suggested that agnoprotein is important for virus assembly, maturation, and/or release (Rinaldo et al., 1998).

Accumulation of late proteins and replicated genomes in the nucleus leads to virion assembly and release of progeny viral particles from the infected cell. The details of assembly and release have not been determined. This is the completion of the viral life

cycle in a permissive cell (Figure 1.2), resulting in production of mature, infectious particles and concluding with lysis of the infected cell or an undetermined mechanism of viral egress to release the progeny virions. In addition, BKV can infect cells that are nonpermissive for productive infection, most notably rodent cells. Nonpermissive infection results in early gene expression (TAg, tAg) but a failure to replicate viral DNA, most likely due to the inability of TAg to interact with and recruit cell replication machinery of another species. The accumulation of TAg and tAg without progression to the late stages of infection results in oncogenic transformation.

BK Virus Persistence and Associated Disease

BKV was first discovered in 1971 in the urine of a renal transplant patient with ureteral stenosis (Gardner et al., 1971). In the same year, JCV was isolated from human brain tissue from a patient with progressive multifocal leukoencephalopathy (PML) (Padgett et al., 1971). From the beginning of their acknowledged existence, the human polyomaviruses were linked to significant disease.

The route of transmission for BKV is not well-defined. It is assumed that primary infection occurs either by the oral or respiratory route because of the widespread nature of BKV infection and the timing of seroconversion: 50% of children are seropositive by the age of 3 and 91% are seropositive by the age of 9 (Knowles et al., 2003). The primary infection is typically subclinical, but has been associated with upper respiratory disease in children (Goudsmit et al., 1982). Approaching adulthood, BKV seroprevalence declines slightly to approximately 81% for the general population (Knowles et al., 2003). Following primary infection, BKV disseminates throughout the

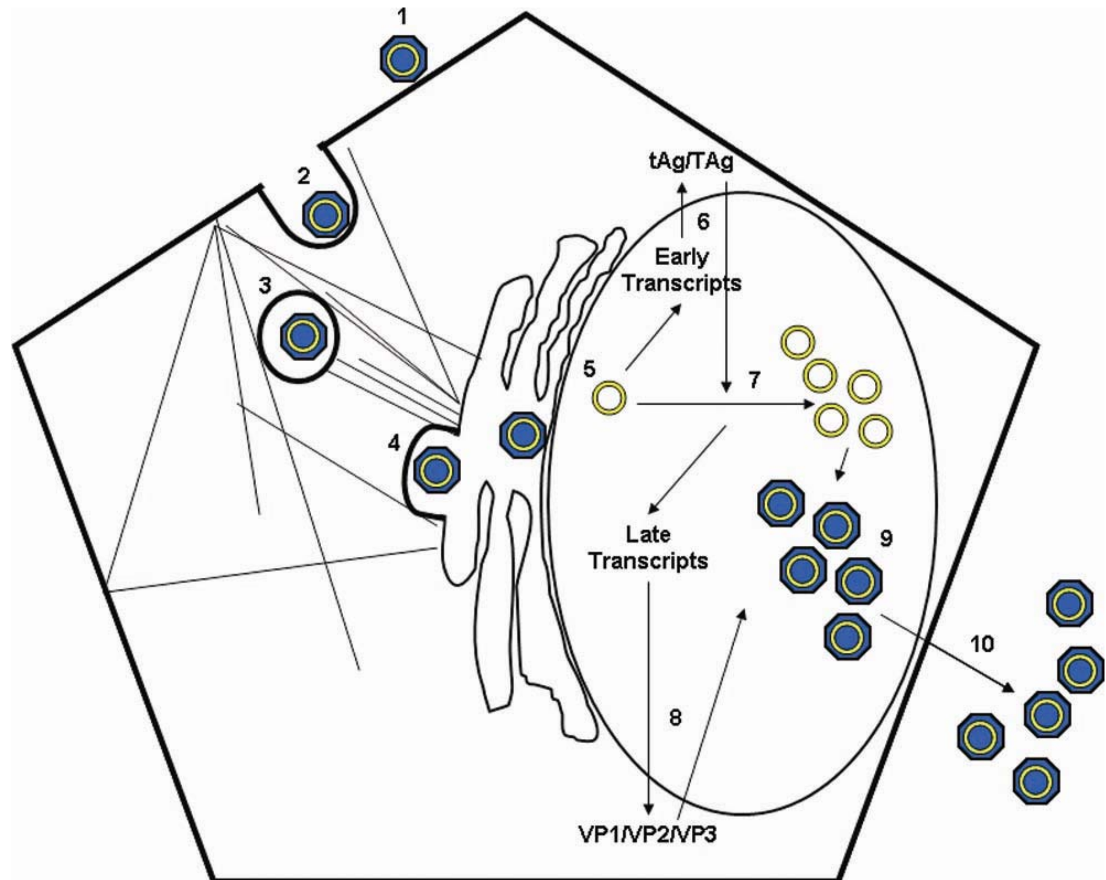

Figure 1.2. The life cycle of BKV. Numbers indicate the steps of BKV infection and replication: 1) binding to receptor GD1b, GT1b, or sialic acid; 2) caveolae-dependent endocytosis; 3) trafficking, which involves microtubules and actin filaments; 4) passage through the endoplasmic reticulum; 5) delivery of genome to the nucleus; 6) early gene transcription and translation; 7) TAg returns to the nucleus to facilitate viral DNA replication and late gene transcription; 8) late gene transcription and translation; 9) capsid proteins return to the nucleus for assembly of progeny virions; 10) release of viral progeny by egress or cell lysis.

body; viral sequences have been reported in a variety of different cell types, including peripheral blood mononuclear cells (Chatterjee et al., 2000; Doerries et al., 1994), liver (Knepper and diMayorca, 1987), bone tissue (De Mattei et al., 1994), and brain (Elsner and Doerries, 1992). Finally, BKV reaches the cells of the kidney and urinary tract, specifically proximal tubule epithelial cells and the urothelium (Chesters et al., 1983; Heritage et al., 1981), where it establishes a persistent, lifelong subclinical infection in healthy individuals. It is reported that 50% of healthy native kidneys and 40% of ureters harbor BKV, with the highest levels (400 genome copies per 100,000 cells) in the renal medulla (Monini et al., 1995; Nickeleit et al., 2003). Approximately 5% of healthy individuals actively excrete BKV in urine (viruria) at a rate of greater than 10^6 genome copies per ml (Zhong et al., 2007). In addition, there is a high tendency of pregnant women to be viruric with no clinical outcome (Coleman et al., 1980; Markowitz et al., 1991). In healthy individuals, BKV has developed an effective means to coexist with the host, maintaining a subclinical infection that sporadically leads to viral excretion in the urine and potential transmission to new hosts.

Certain conditions of the host, specifically immunosuppression, can allow BKV to 'reactivate' from the persistent subclinical state to an active lytic infection, resulting in serious disease. This is a feature common to polyomaviruses, as JCV, Py, and SV40 all undergo reactivation in immunocompromised hosts (Imperiale and Major, 2007). Two major patient groups are highly susceptible to BKV reactivation: bone marrow transplant (BMT) recipients and kidney transplant (KT) recipients. In BMT patients, BKV lytic infection occurs in the epithelial cells of the bladder or ureter, resulting in hemorrhagic cystitis (HC) characterized by viruria and hematuria. HC is reported in 10 to 68% of

BMT patients, with 25% suffering from severe HC with heavy bleeding and intense pain (Arthur et al., 1986; Azzi et al., 1994; Bedi et al., 1995; Cotterill et al., 1992). BKV-associated HC occurs more than 10 days post-transplant (late-onset) and requires medical attention but is not usually life-threatening (Egli et al., 2007). It has been suggested that HC is an immune reconstitution syndrome, in which the reactivation of BKV, and thus the presence of large amounts of viral antigens, sets off an excessive inflammatory response that causes damage to the urothelial cells (Hirsch, 2005).

In KT recipients, reactivation of BKV is first evident by viral shedding in the urine, which occurs in 20 to 60% of patients. About half of these patients then develop high levels of viremia (virus dissemination in the bloodstream; Brennan et al., 2005; Hirsch, 2005; Hirsch et al., 2002). In these patients, BKV that established a subclinical infection in kidney epithelial cells has reactivated to a productive lytic infection in which viral progeny have spread to the surrounding cells of the medulla, renal cortex, and proximal tubules. This condition is referred to as polyomavirus nephropathy (PVN) and the end stage involves necrosis of the tubules and denudation of the basement membrane (Nickeleit et al., 2003). PVN is diagnosed primarily by examination of kidney biopsies for characteristics of virus-infected cells (enlarged nuclei, cytopathic effects), followed by immunohistochemistry to detect TAg expression (Drachenberg et al., 2005; Vats et al., 2006). In addition, PCR quantitation of viral genomes in biopsy samples and urine cytology to identify decoy cells (BKV-infected cells with characteristic morphology) are often employed (Singh et al., 2006). If not controlled, the lytic infection will cause failure and destruction of the graft. Approximately 1 to 10% of KT patients develop PVN, primarily during the first year post-transplantation, which coincides with the time

of highest immunosuppressive treatments. Once diagnosed with PVN, 50 to 90% of patients progress to graft failure (Egli et al., 2007). The incidence of PVN is increasing, most likely due to the combined effects of more potent immunosuppressive therapies, better methods of diagnosis, and the increasing number of KT performed every year.

The treatment options for PVN are quite limited. The most effective treatment to date is reduction of the immunosuppressive regimen, which allows the patient to mount an immune response and fight the infection. There are currently no proven antiviral treatments for BKV, although some limited success has been seen with cidofovir and leflunomide (Bernhoff et al., 2008; Vats et al., 2006). A third approach is nephrectomy to remove the transplanted kidney. This has been shown to cause a rapid decrease in viral load, with a viral half-life as fast as one to two hours and greater than 99% turnover each day, compared to a viral half-life of six hours to 17 days resulting from a decrease in immunosuppression (Funk et al., 2006). These findings suggest that the graft is the source of replicating BKV, not the kidney epithelial cells or urothelium of the recipient.

There is no single risk factor shared by all patients that develop PVN; it is thought that BKV reactivation is prompted by a combination of factors from the virus, patient and graft (Comoli et al., 2006; Egli et al., 2007; Hirsch et al., 2006). Specific individual risk factors include older age, male gender, seropositivity of the donor, seronegativity of the recipient, specific immunosuppressive drugs, HLA mismatches, and acute rejection episodes prior to development of PVN (Andrews et al., 1988; Bohl et al., 2005; Comoli et al., 2006; Egli et al., 2007; Mengel et al., 2003). It is clear that an immunosuppressed state alone is not enough to cause PVN; not all KT patients suffer from PVN and there are only sporadic references to BKV reactivation and associated nephropathy in other

solid organ transplant patients or immunodeficiency syndromes, such as AIDS (Hirsch, 2005; Pavlakis et al., 2006).

Therefore, a primary topic addressed by investigators currently studying BKV reactivation is the identification of component(s) of the immune system that are critical for controlling BKV infections. Further knowledge about the immune response to and control of BKV in healthy individuals may allow better treatment options for patients with PVN and HC. Several clinical observations have been made about the immune response during BKV reactivation. It is reported that 77% of patients are seropositive for BKV before KT (Hirsch et al., 2002) and that patients with detectable anti-BKV antibodies still progress to PVN (Comoli et al., 2004). In fact, it seems that antibodies are more indicative of the viral load in a patient rather than protective against BKV infection, as high levels of antibodies in PVN patients correlate with high levels of viremia and low $CD8^+$ T cell responses (Chen et al., 2006). Thus, anti-BKV antibodies do not appear to play a major role in controlling infection and reactivation. Furthermore, the cell-mediated cytotoxic immune response fails to eliminate all BKV-infected cells, allowing the establishment of a persistent infection in healthy individuals. Cytokine-producing T cells now show the most promise as key regulators of BKV reactivation: low levels of BKV-specific IFN-γ-producing T cells correlate with progression to PVN, while reconstitution of these cells correlates with resolution of PVN (Binggeli et al., 2007; Chen et al., 2006; Prosser et al., 2008). Therefore, the fluid-borne effectors produced by T cells, such as cytokines, may be a critical means to control BKV replication and reactivation.

BKV NCCR and Transcriptional Regulation

BKV strains can be divided into two major types, archetype and rearranged, based on the structure of the NCCR. The archetype NCCR has a simple structure composed of five blocks of nucleotide sequences, designated O, P, Q, R, and S (Figure 1.3; Markowitz and Dynan, 1988; Rubinstein et al., 1987; Sundsfjord et al., 1994). Rearranged strains have major structural changes in the NCCR, relative to the archetype, involving partial or whole duplications, deletions, or mutations of these blocks. The O, P, Q, R, and S blocks were defined as sequences that appear to move together when rearrangements occur. The O block is 142 bp long and highly conserved among all strains of BKV, likely because it contains the origin of replication, the TATA box, and the three TAg binding sites that are required to initiate replication. The P block is 68 bp long and is present at least once in every strain isolated thus far, though it is frequently duplicated in part or in whole (Moens and Van Ghelue, 2005). This region contains a cAMP response element (CRE) and proven binding sites for the transcription factors Sp1, AP-1, and NF-1. The Q block is 39 bp long and contains proven binding sites for Sp1, NF-1, and NF-κB. The R block is 63 bp long and has only been shown to contain an NF-1 binding site. The S block, leading up to the start codon of agnoprotein, is 63 bp long and contains two proven NF-1 sites, an estrogen response element, and a glucocorticoid and/or progesterone response element. The Q and R blocks are frequently deleted from rearranged strains, while the S block is rarely altered (Cubitt, 2006; Johnsen et al., 1995; Moens and Rekvig, 2001; Moens and Van Ghelue, 2005).

Rearrangements of the NCCR can add or remove transcription factor binding sites either by the addition or deletion of blocks that contain these sites, or by splicing

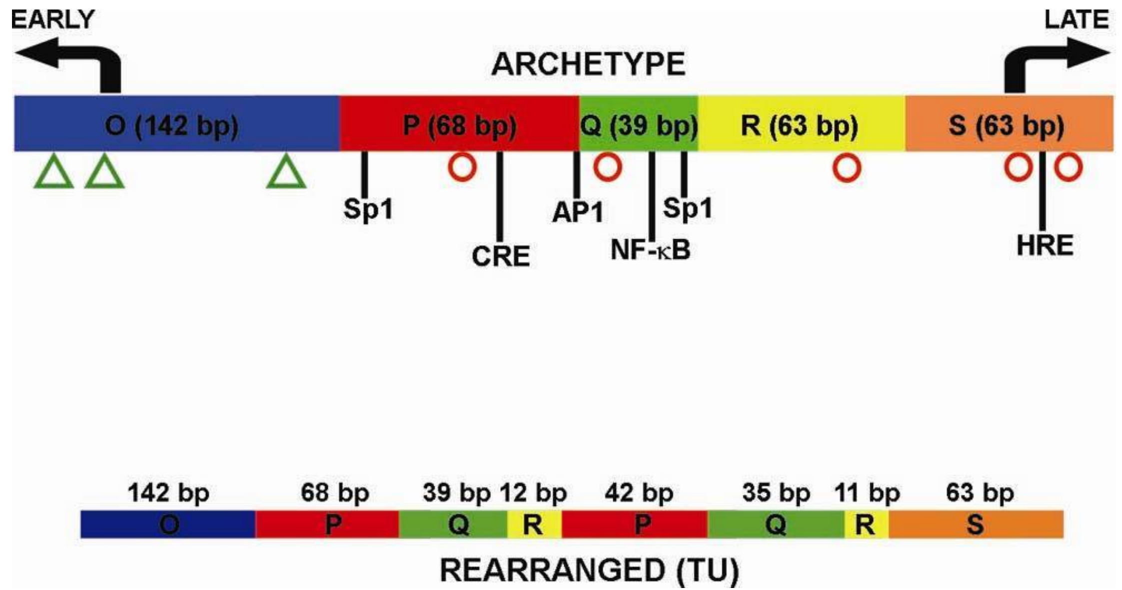

Figure 1.3. The structure of archetype NCCR. A schematic representation of the structure of archetype BKV NCCR. Blocks of transcription factor binding sites are labeled by letter and length. The locations of binding sites discussed in the text are shown. Open green triangles represent TAg binding sites. Open red circles represent NF-1 binding sites. Arrows indicate the bidirectional nature of the promoter, initiating early gene transcription to the left and late gene transcription to the right. Below is a schematic representation of the structure of a rearranged NCCR, BKV TU, which is used in most of our experiments. CRE, cAMP response element; HRE, hormone response element.

sequences together, creating new binding sites at the junctions of blocks (Markowitz and Dynan, 1988; Markowitz et al., 1990). Furthermore, BKV promoters may be regulated differently depending on the cell type infected. It has been shown by DNase footprint analysis that different protein complexes bind to the NCCR during incubation with HeLa versus 293 cell extracts (Grinnell et al., 1988). The observed binding patterns correlate with differences in promoter activity in these two cell lines, suggesting that replication capacity may also differ in a cell type-dependent manner.

From the beginning, it was clear that there is a functional difference between rearranged and archetype NCCRs. Archetype viruses are more efficient at transforming rodent cells but extremely inefficient at propagation in tissue culture, while rearranged BKV strains are highly adapted to growth in tissue culture but unable to efficiently transform cells (Watanabe and Yoshiike, 1982; Watanabe and Yoshiike, 1986). Upon examination of the individual blocks, it was observed that an NCCR with a single P block was enough to initiate transcription, but that repetitions of the P block resulted in greater promoter activity (studies in HeLa cells; Chakraborty and Das, 1989; Deyerle and Subramani, 1988). Insertion of P block fragments of a rearranged NCCR into an archetype NCCR resulted in enhanced transcriptional activity, indicating that it is not the presence of repressor elements in archetype strains, but instead the duplication of activator elements in rearranged strains that results in the elevated capacity to replicate (Markowitz et al., 1990). By comparing the growth phenotypes of different BKV strains to their NCCR structures, it was noted specifically that the duplication of P blocks favors growth in HeLa cells (Markowitz and Dynan, 1988). It was also shown that, in the presence of HeLa cell extracts and immunoaffinity purified TAg, a plasmid containing an

NCCR with a single P block replicated as well as one with three adjacent P blocks, and that mutation of the NF-1 and Sp1 binding sites within the P block had no effect on replication (Del Vecchio et al., 1989). These results suggest that, in the presence of abundant TAg, the transcription and replication elements of the NCCR are separable; however, the functions *in vivo* are not, since replication requires efficient TAg expression, implying that the greater the activity of the early promoter, which drives the expression of TAg, the greater the replication capacity of the virus.

It has long been thought that NCCR structure would play a role in BKV pathogenesis by giving rearranged viruses selective replication advantage over the archetype strains. In many independent experiments, an archetype strain from a clinical isolate showed essentially no replication in tissue culture over the course of many weeks. If viral replication was detected, the NCCR structure of the progeny virus was rearranged, indicating that either a rearrangement event had occurred or the clinical sample used for infection contained an initially undetectable level of rearranged virus that eventually outgrew the archetype (Rinaldo et al., 2005; Rubinstein et al., 1991; Sundsfjord et al., 1994; Sundsfjord et al., 1990). These studies have been performed in human umbilical vein endothelial (HUVEC-C), monkey kidney epithelial (Vero), and human embryonic kidney (HEK) cell lines. A similar phenomenon is seen when archetype genomic DNA is transfected into cells: after extended incubation, the progeny virus, if produced, has NCCR rearrangements (Rinaldo et al., 2005; Rubinstein et al., 1991). This result directly demonstrates the evolution of a rearranged strain of BKV from an archetype genome.

Despite the evidence that rearranged strains are better at replicating in tissue culture, the vast majority of strains isolated from both healthy individuals and immunosuppressed patients have archetype NCCRs (Gosert et al., 2008; Markowitz et al., 1991; Negrini et al., 1991; Sharma et al., 2007; Sugimoto et al., 1989; Sundsfjord et al., 1999; Takasaka et al., 2004; ter Schegget et al., 1985). It was recently reported, however, that rearranged strains are found more frequently in the blood of patients with PVN than in the urine, and that these strains attain 20-fold higher viral loads than archetype strains in the blood (Gosert et al., 2008). In addition, kidney biopsies from PVN patients with rearranged strains had more evidence of inflammation than those with archetype strains. This is the first report to link rearranged strains directly to more extensive viral replication and subsequent disease in patients. Still, the authors could not identify any commonalities in the rearrangements that would implicate a specific NCCR element in pathogenesis. The authors hypothesized that archetype strains are more successful in immunocompetent hosts because of their ability to remain relatively undetected by the immune system, by means of slower growth and lower levels of TAg (Gosert et al., 2008). While the clinical relevance of NCCR rearrangements is not yet clear, many researchers are trying to show the association between transcription factor binding sites or NCCR structures with progression to disease. For example, mutations in the Sp1 binding site at the junction of the Q and R blocks may be found more frequently in BMT patients with HC than those without, but the relevance and effect of the mutation on viral replication is not known (Priftakis et al., 2001).

Interferon-Gamma and Polyomaviruses

Interferons are a class of cytokines widely known for their antiviral effects, and in fact were first discovered and named for their ability to "interfere" with viral infection (Isaacs and Lindenmann, 1957; Wheelock, 1965). There are three classes of interferons, type I, type II, and type III, related by function and signaling pathways (reviewed in Biron and Sen, 2001; Pestka et al., 2004). Type I interferons are a constantly growing class of molecules, which currently include IFN-α, IFN-β, IFN-ε, IFN-δ, IFN-κ, IFN-τ, IFN-ω, and the interferon-like cytokine limitin. Type III interferons, or IFN-λ, were only recently discovered and have two members, IL-28 and IL-29 (Ank et al., 2006). IFN-γ is the only member of the type II class, distinct from type I in that it is produced primarily by immune cells, mainly natural killer (NK) cells, T cells, antigen presenting cells (APCs), and B cells. Type I interferons play a major role in innate immunity against viruses, as they are produced by almost all cell types and are quickly induced by viral components, such as double-stranded RNA. In contrast, IFN-γ can be produced as an innate immune mediator by NK cells, or as an adaptive immune mediator when produced by T cells, B cells, and APCs. Viral antigens must be presented to T cells in the proper MHC context to activate IFN-γ expression. IFN-γ is primarily associated with the T helper 1 (Th1) phenotype: production is enhanced by IL-2, IL-1, estrogen, IL-18, IL-12, and IFN-γ itself, but inhibited by glucocorticoids, TGF-β and cytokines associated with the Th2 phenotype (Biron and Sen, 2001).

IFN-γ is a 21 kDa cytokine expressed as a homodimer. The receptor is present on the surface of almost all nucleated cells and is composed of two heterodimers of IFNGR1

and IFNGR2 molecules, distinct from the IFN-α/β receptor. Janus kinase 1 (JAK1) is bound to the cytoplasmic tail of IFNGR1 while JAK2 is bound to IFNGR2, and upon IFN-γ binding, these two kinases are phosphorylated. JAK1 and JAK2 then phosphorylate the cytoplasmic domain of IFNGR1, which allows binding of signal transducer and activator of transcription 1 (STAT1) to the receptor. STAT1 molecules are then phosphorylated which results in their dimerization; homodimers of STAT1, known as the gamma-activated factor (GAF), translocate to the nucleus and bind gamma-activated sequences (GAS) to promote transcription of IFN-γ responsive genes. Crosstalk between the IFN-α/β and the IFN-γ signaling cascades can result from IFN-α/β-stimulated activation of GAF, although primarily the type I interferons signal through the complex of STAT1, STAT2, and interferon regulatory factor 9 (IRF-9), which binds the interferon-stimulated response element (ISRE) in promoters. Furthermore, in certain situations GAF can complex with IRF-9 and mediate signaling through ISREs (reviewed in Pestka et al., 2004; van Boxel-Dezaire and Stark, 2007).

The effects of IFN-γ signaling on a cell are far-reaching and diverse (Biron and Sen, 2007; Sen, 2001; van Boxel-Dezaire and Stark, 2007). First, IFN-γ has an important immunomodulatory role which it mediates by activating monocytes to produce nitric oxide synthase 2 (iNOS, important for eliminating bacteria), inducing expression of chemokines to recruit and activate immune cells, activating NK cell cytotoxicity, increasing MHC class I and II expression, promoting differentiation of $CD4^+$ T cells, and promoting immunoglobulin class switching. In nonimmune cells, interferon signaling is mostly directed at establishing an antiviral state. Among the genes activated are protein kinase R (PKR), which phosphorylates eukaryotic initiation factor 2 (eIF-2α) to inhibit

translation; 2’-5’ oligoadenylate synthetase, which activates RNaseL to nonspecifically degrade mRNA; adenosine deaminase, which introduces adenosine to inosine changes in transcripts to affect protein expression and function; and IRFs, which potentiate the signaling cascade by mediating interferon synthesis and interferon responsive gene regulation. The overall effect on the cell is anti-proliferative and pro-apoptotic. Transcription factors other than STAT1 known to be activated during IFN-γ signaling include STAT3, AP-1, USF-1, NF-κB, IRF-1, IRF-8, ATF-2, GATA-1, CREB, and PU.1, as well as C-EBP-β and CIITA, which are newly synthesized in response to signaling and mediate the second wave of the cascade (van Boxel-Dezaire and Stark, 2007). The crosstalk between the type I and type II interferon pathways creates difficulty in defining genes as being specifically regulated by IFN-α/β or IFN-γ. Several studies have examined the differential effects of interferon signaling by microarray in various cell types, including human lung carcinoma (Sanda et al., 2006; Tan et al., 2005) and human fibrosarcoma (Der et al., 1998) cell lines.

Many viruses have developed methods to overcome the interferon response by blocking interferon synthesis or interferon-mediated signaling and effectors, or even by expressing interferon receptor decoys (Biron and Sen, 2007; Sen, 2001). Despite the small genome size, and thus the limited ability to encode specific inhibitor and decoy proteins, there are several reports that polyomaviruses can resist the antiviral effects of interferons. SV40 TAg can inhibit PKR-mediated deactivation of eIF-2α, most likely by stimulating a cellular phosphatase to dephosphorylate eIF-2α (Swaminathan et al., 1996). Py has been shown to specifically inhibit IFN-β-stimulated gene expression by blocking

the activation of the STAT1/STAT2/IRF-9 complex through the interaction of TAg with JAK1 (Weihua et al., 1998).

The regulation of human polyomaviruses by interferons is currently being investigated. With the increasing popularity of microarray studies, two groups have separately examined changes induced by JCV infection of cultured cells. The first study analyzed the effect of infection with JCV/SV40 chimeric viruses, containing JCV coding regions and JCV/SV40 hybrid NCCRs, on human fetal astrocyte cultures. During infection, there was a strong upregulation in cell cycle genes, as anticipated, but surprisingly no effect on interferon-responsive genes and very little effect on genes relating to the immune response in general (Radhakrishnan et al., 2003). In contrast, the second study reported that transfection of JCV genomic DNA into primary human fetal glial cells stimulated a variety of immune response genes, in particular interferon-stimulated genes (Verma et al., 2006). The upregulation of several factors was confirmed during JCV infection and, in a follow-up study, the authors reported that the induction of the interferon response required active JCV replication, not just the presence of viral products or DNA (Co et al., 2007). In addition, they noted an inhibition of JCV replication by IFN-α/β treatment at late time points during infection (Co et al., 2007). There has only been one microarray study published analyzing the effect of BKV infection on immortalized human umbilical vein endothelial cells. As expected, the authors report the upregulation of cell cycle-related genes at various times after infection; however, they saw no induction of interferon transcripts, the upregulation of only two interferon-inducible genes, and the downregulation of several genes involved in antiviral defense (Grinde et al., 2007). These results indicate that BKV may have the ability to

infect cells in such a way as to avoid detection by the innate intracellular immune response.

Transforming Growth Factor-Beta

Members of the TGF-β family of cytokines are secreted proteins that share sequence and structural features and have similar signaling cascades within the cell. This family includes the bone morphogenic proteins (BMPs), which are important during development; activins, which regulate growth and differentiation; and the TGF-β isoforms, TGF-β1, TGF-β2, and TGF-β3, which have similar functions in the adaptive immune response. The TGF-β family members are expressed in most cell types, although the effect on TGF-β signaling can be cell type-dependent. This section will focus specifically on the details of TGF-β1 signaling and regulation.

The precursor of TGF-β is a prepro-peptide that contains a signal peptide for secretion, an immature propeptide called the latency associated protein (LAP) that is removed by proteolytic cleavage in the Golgi (Dubois et al., 1995), and the mature TGF-β protein, a 25 kDa dimer (Feng and Derynck, 2005; Li et al., 2006; Rahimi and Leof, 2007). TGF-β can be secreted in two different forms: the small latent complex (SLC), a homodimer of mature TGF-β associated with a homodimer of LAP, or the large latent complex (LLC), the SLC in association with latent-TGF-β binding protein (Annes et al., 2003). The liberation of mature TGF-β from the latency proteins *in vivo* is not fully understood, but can be mediated by thrombospondin-1, matrix metalloproteinases, integrins and reactive oxygen species, among others (Li et al., 2006; Yang et al., 2007). The mature TGF-β binds to a cell surface receptor that is composed of two type I

receptors (TβRI) and two type II receptors (TβRII). Upon ligand binding, the receptor complex autophosphorylates: first TβRII phosphorylates TβRI and then TβRI itself undergoes autophosphorylation. Finally, TβRI phosphorylates Smad proteins in the cytoplasm (Derynck and Zhang, 2003; Shi and Massague, 2003).

There are eight Smad proteins expressed in mammalian cells, falling into three classes: the receptor Smads (R-Smad, Smad1, 2, 3, 5, 8), the common Smad (Co-Smad, Smad4), and the inhibitory Smads (I-Smad, Smad6, 7). R-Smads (Smad2 and 3 in the case of TGF-β1) are phosphorylated by TβRI, form a complex of two R-Smads and one Co-Smad, and then translocate to the nucleus to act as transcription factors. This Smad2/3/4 complex binds to (5' CAGAC 3') sequences in promoters, also known as the Smad binding element (SBE; Shi et al., 1998; Zawel et al., 1998). Smad proteins by themselves have only weak DNA binding activity unless multiple SBEs are present (Shi et al., 1998; Zawel et al., 1998). More commonly, the complex interacts with other transcription factors that have more specific or higher affinity DNA binding abilities to regulate gene expression. These factors must have the ability to interact with Smad proteins and have a DNA binding site nearby to the SBE (Derynck and Zhang, 2003; Shi and Massague, 2003). Co-Smad interactions help to stabilize the R-Smad interactions with other transcription factors. I-Smads are negative regulators of Smad signaling: Smad6 competes with Smad4 for binding to R-Smads and forms inactive complexes (Hata et al., 1998), while Smad7 competes with R-Smads for binding to the activated receptors (Hayashi et al., 1997; Nakao et al., 1997). Smad7 is induced by IFN-γ signaling through the JAK/STAT pathway and by NF-κB signaling in response to inflammatory cytokines (Bitzer et al., 2000; Giannopoulou et al., 2006; Ulloa et al.,

1999). There is an extensive list of transcriptional coactivators and corepressors that interact with Smad complexes and mediate TGF-β-responsive gene regulation, including AP-1, FAST proteins, NF-κB, p300/CBP, P/CAF, Sp1, and ZEB proteins (Brown et al., 2007; Feng and Derynck, 2005).

The effects of TGF-β family members vary depending on the cell type, but overall these cytokines can regulate proliferation, differentiation, migration, and cell survival during development, carcinogenesis, fibrosis, wound healing and immune responses (Blobe et al., 2000; Li et al., 2006). In immune cells, TGF-β-mediated signaling generally results in inhibition of growth and differentiation, resulting in tolerance and immunosuppressive effects (Li et al., 2006). Similarly, TGF-β signaling in epithelial cells usually results in growth arrest and apoptosis; in fibroblasts, however, TGF-β promotes proliferation and activation (Rahimi and Leof, 2007). Several reports have shown that various immunosuppressive therapies commonly used in both solid organ transplant and BMT patients cause an increase in TGF-β expression, particularly in renal epithelial cells (Khanna et al., 1999a; Khanna et al., 1999b; McMorrow et al., 2005; Shihab et al., 1996); these observations complement the overall immunosuppressive effects of TGF-β.

There are numerous reports of viruses that induce TGF-β expression, including cytomegalovirus, hepatitis B virus, hepatitis C virus, and HIV; the immunosuppressive effects of TGF-β have obvious benefits for the virus during infection (Li et al., 2006; Reed, 1999). However, there are also several reports of viruses that are regulated by the signaling events of the TGF-β pathway. TGF-β treatment was shown to inhibit viral RNA replication and protein expression from a hepatitis C virus replicon (Murata et al.,

2005). Overexpression of Smad3 and Smad4 proteins regulates the activity of HIV-1 LTR in astrocytes (Coyle-Rink et al., 2002). TGF-β1 can induce the reactivation of lytic EBV replication from latently-infected epithelial cells (Fukuda et al., 2001) and B cells (di Renzo et al., 1994; Fahmi et al., 2000). In addition, JCV early and late promoter activities are upregulated by Smad3 and Smad4 overexpression (Enam et al., 2004), and treatment of JCV-infected astrocytes with TGF-β stimulates viral replication (Ravichandran et al., 2007). Therefore, TGF-β expression and regulation is highly relevant for viral infections.

Summary and Chapter Outline

The human polyomavirus BKV is able to establish a persistent, subclinical infection in nearly the entire population, which demonstrates its ability to at least partially evade the cytotoxic immune response. In immunosuppressed patients, particularly KT and BMT recipients, BKV can reactivate to a productive lytic infection, suggesting that the loss of certain immune components in conjunction with other factors alleviates repression of viral infection. We hypothesized that soluble immune factors, such as cytokines, may play an important role in regulating BKV. Therefore, the decrease in lymphocyte proliferation and activation during immunosuppression may promote BKV reactivation, as a result of reduced cytokine expression.

Previously, our lab has developed a cell culture system for the study of BKV infection in the natural host cell for reactivation, renal proximal tubule epithelial cells (Low et al., 2004). In this system, the absence of immune surveillance mimics the environment of an immunosuppressed patient and may allow the examination of cytokine-mediated effects on BKV in a highly relevant host cell. In Chapter II, we

describe the inhibitory effect of IFN-γ on BKV replication and demonstrate that the primary level of regulation is early gene transcription. In Chapter III, we report the differential effects of TGF-β on early promoter activity. In contrast to IFN-γ-mediated regulation, which is similar for three rearranged strains of BKV examined, TGF-β-mediated effects vary, depending on the strain of virus and the cell type. In Chapter IV, we begin a deeper investigation to analyze the mechanism of BKV promoter regulation, specifically chromatin remodeling, in the context of IFN-γ restriction of replication and during infection with archetype virus. In Chapter V, we will discuss the relevance of our findings and the overall future directions of this project.

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CHAPTER II

INHIBITORY EFFECT OF INTERFERON-GAMMA ON BK VIRUS GENE EXPRESSION AND REPLICATION

Polyomavirus nephropathy (PVN) results in renal dysfunction and graft loss in up to 10% of all kidney transplant recipients (Hirsch et al., 2005). It is widely accepted that BK virus (BKV) is the etiological agent responsible for the majority of cases of PVN, which are typically diagnosed within the first year after transplantation (Hirsch et al., 2002; Hirsch and Steiger, 2003). PVN is characterized by the lytic, destructive replication of BKV in proximal tubule epithelial cells in the transplanted kidney and is normally diagnosed by renal biopsy to assess histological effects of infection, PCR to determine viral presence and loads in the urine and blood, and the detection of decoy cells, which are cells with distinct intranuclear inclusion bodies that are shed during active BKV replication, in the urine (Drachenberg et al., 2005; Hirsch, 2005; Nickeleit et al., 2003). Since there are currently no effective antiviral treatments for BKV infection, the most common approach used to control PVN is to decrease the patient's immunosuppressive regimen. However, such an approach increases the risk of graft rejection and thus is not an appealing strategy. The prevalence of PVN is increasing with the advent of new, more powerful immunosuppressive therapies, making it a growing concern for the transplant community.

The human polyomavirus BKV was first isolated in 1971 in the urine of a renal transplant recipient (Gardner et al., 1971). BKV virions are small (40 to 45 nm in diameter), non-enveloped, icosahedral, and contain a circular, double-stranded DNA genome of approximately 5.2 kb (Moens and Rekvig, 2001), which is associated with cellular histones to form a chromatin-like structure (Meneguzzi et al., 1978). The genome encodes only six known proteins: the early proteins, large tumor antigen (TAg) and small tumor antigen (tAg), and the late proteins, VP1, VP2, VP3, and agnoprotein. BKV infects nearly the entire population, with seroprevalence reaching 60 to 80% by the age of 10 (Knowles, 2001). BKV is thought to be contracted by respiratory transmission and the primary infection is typically subclinical. Following the initial infection, BKV spreads to other cells of the body, most notably peripheral blood mononuclear cells (Doerries et al., 1994) and cells of the kidney and urinary tract (Chesters et al., 1983; Heritage et al., 1981; Shinohara et al., 1993), in which the virus establishes a persistent, subclinical infection. It is from these sites in immunocompromised patients that BKV reactivates to a lytic infection, resulting in BKV-associated diseases such as PVN.

Previously, we described an *in vitro* system that allows the study of BKV lytic infection of primary human proximal tubule epithelial cells (Low et al., 2004). The functions of proximal tubule cells in the kidney include facilitating the recovery of blood products, maintenance of blood pressure and volume, and production and release of cytokines and chemokines to communicate with the host immune system (Briggs et al., 2001; Daha and van Kooten, 2000). Proximal tubule cells remain in a differentiated state for up to six passages in tissue culture (Humes et al., 2002) and thus provide an environment similar to that which BKV encounters in an immunocompromised host. By

introducing individual elements of the immune system to this model, we can begin to determine which components regulate BKV replication. In part because proximal tubule epithelial cells are known to interact both with neighboring cells and with the immune system through the production of cytokines and chemokines, namely IL-6, IL-8, IL-15, TNF-α, MCP-1, RANTES, and TGF-β (Daha and van Kooten, 2000), we began to investigate the role of cytokines in mediating regulation of BKV replication.

Our interest in the effects of cytokines on BKV replication was initiated by clinical reports and observations that argued for the importance of the cell-mediated immune response, as opposed to other arms of the immune system, in controlling BKV persistent infection. First, it has been demonstrated that up to 90% of the adult population has BKV-specific antibodies (Knowles, 2001) and patients with levels of anti-BKV antibodies similar to those of healthy individuals still develop PVN (Hariharan et al., 2005; Hirsch et al., 2002). Several reports detail the activation of the humoral immune response in patients with PVN, although antibody levels are shown to increase only after the stabilization of renal function and a decrease in viral load (Chen et al., 2006; Comoli et al., 2004; Hariharan et al., 2005). Furthermore, high titers of BKV-specific antibodies in the donor correlate well with the incidence of BKV reactivation in the recipient (Bohl et al., 2005). These findings suggest that the presence of BKV-specific antibodies does not prevent reactivation and development of PVN, although they may be effective at controlling viremia (Chen et al., 2006). In addition, the failure of the cytotoxic T lymphocyte (CTL) response to eliminate all cells harboring BKV, as demonstrated by the well-documented periodic shedding of virus in immunocompetent individuals, indicates that another facet of the immune system plays a role in controlling

persistent infection. There are several studies demonstrating the correlation of low levels of BKV-specific interferon-gamma (IFN-γ)-producing cells and the development of viremia and PVN (Chen et al., 2006; Comoli et al., 2004). Furthermore, others have reported the inhibitory effect of IFN-γ on the promoters of SV40, a related polyomavirus, and human cytomegalovirus, which lacks structural relatedness to BKV but has similar epidemiological features, such as latency in the kidney and reactivation upon immunosuppression (Harms and Splitter, 1995; Qin et al., 1997; Ritter et al., 2000). These findings prompted us to investigate the potential regulation of BKV replication by IFN-γ.

IFN-γ is the sole member of the type II family of interferons and is a secreted glycoprotein of ~25 kDa produced primarily by natural killer cells during the innate immune response and specific antigen activated-T lymphocytes during the adaptive immune response (Pestka et al., 2004; Schroder et al., 2004). Activation of the IFN-γ cascade within a cell is initiated by the binding of IFN-γ to the cell surface receptor and subsequent activation of the JAK-STAT signaling pathway. JAK1 and JAK2 kinases, which are associated with the two chains of the IFN-γ receptor, IFNGR1 and IFNGR2, respectively, become phosphorylated upon IFN-γ binding. As a result, STAT1 homodimers are phosphorylated and subsequently translocated to the nucleus, where they act as transcription factors to mediate regulation of IFN-γ responsive genes (Pestka et al., 2004; Schroder et al., 2004). IFN-γ was first discovered by its antiviral activity (Wheelock, 1965) and remains widely known as a potent antiviral cytokine. Among the genes up-regulated by the IFN-γ signaling cascade are protein kinase R, which inhibits cellular translation by phosphorylating and inactivating eukaryotic initiation factor-2; 2'-

5' oligoadenylate synthetase, which activates RNaseL to non-specifically degrade mRNA transcripts and inhibit gene expression; and interferon regulatory factor 1 (IRF-1), which up-regulates production of caspase 1 and promotes apoptosis of the cell. IFN-γ also plays a major role in the activation and recruitment of immune cells and up-regulates the expression of MHC class I and II molecules on the cell surface. More generally, IFN-γ significantly (by at least two-fold) affects the expression of more than 100 genes in mammalian cells (Der et al., 1998). In addition, other genes may be regulated indirectly by a group of IFN-γ-responsive transcription factors that perpetuate the signaling cascade. These transcription factors may also act on viral promoters to inhibit viral gene expression and replication.

In this report, we characterize the specific inhibitory effect of IFN-γ on BKV gene expression and replication during lytic infection of proximal tubule cells. We were interested in determining the points at which the viral life cycle is affected and the conditions in which IFN-γ has the strongest inhibitory effect on viral replication. IFN-γ inhibited BKV gene expression, both at the level of transcription and translation, and reduced the level of viral progeny produced during lytic infection. These results are important for understanding the host immune response to BKV and, more specifically, the role of cytokines in regulating BKV replication and infection.

Materials and Methods

Cell culture. Primary human renal proximal tubule epithelial cells (RPTE cells, Cambrex) were maintained for up to six passages in renal epithelial cell basal medium (REBM, Cambrex) supplemented with human epidermal growth factor, fetal bovine serum, hydrocortisone, epinephrine, insulin, triiodothyronine, transferrin, and GA-1000 as indicated for renal epithelial cell growth medium (REGM, supplements obtained as REGM SingleQuots, Cambrex). RPTE cells were grown at 37°C with 5% CO2 in a humidified incubator.

Viruses. The genome of the TU strain of BKV was cloned into the EcoRI site of pGEM-7Zf(-). Genomes of the Dunlop and Proto-2 strains of BKV were cloned into the BamHI site of pBR322 (gift of P. M. Howley). BKV stocks were prepared from these genomic clones: 4 μg plasmid DNA was digested with restriction enzymes (EcoRI for BKV(TU), BamHI for BKV(Dunlop, Proto-2)), recircularized with T4 DNA ligase and phenol-chloroform extracted, and the resulting DNA was transfected into one T75 flask of 60% confluent RPTE cells (~ 4 x 10^6 cells) using Effectene (Qiagen). After three weeks, cells and supernatants were collected and viral lysates were prepared by three freeze (-80°C) / thaw (37°C) cycles. The resulting lysates were used to infect four T75 flasks of 70% confluent RPTE cells and after three weeks, viral lysates were prepared as above. The resulting viral stocks were titrated by fluorescent focus assay and the integrity of the non-coding control region (NCCR) was confirmed by sequencing of PCR products.

Cytokines and chemokines. Recombinant human IFN-γ, IL-6, IL-8, MCP-1, RANTES, and TNF-α were purchased from PeproTech, Inc. and reconstituted according to manufacturer's recommendations. Recombinant human IFN-α was purchased from

Sigma and supplied as a solution in PBS. The doses used to treat BKV-infected RPTE cells were as follows: IL-6, IL-8, MCP-1, TNF-α were used at 100 ng/ml, RANTES was used at 300 ng/ml, IFN-α was used at 50 or 250 U/ml, and IFN-γ was used primarily at 50 or 250 U/ml, with the exception of the dose response experiment in which six five-fold dilutions were used, starting from 1250 U/ml.

Infections. Unless otherwise stated, 70% confluent RPTE cells were infected with the TU strain of BKV at an MOI of 0.5, incubating for one hour at 37°C. Viral lysate used for the infection was replaced with fresh media (REGM) and cytokines were added three to six hours post-infection.

Western blotting. Unless otherwise stated, total cell protein was harvested at four days post-infection using E1A lysis buffer (Harlow et al., 1986) supplemented with 5 μg /ml PMSF, 5 μg /ml aprotinin, 5 μg /ml leupeptin, 0.05 M sodium fluoride, and 0.2 mM sodium orthovanadate. The Bio-Rad protein assay was used to determine the protein concentration of each lysate and 8 μg of protein were electrophoresed on an 8% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membrane in 1x Towbin buffer (25 mM Tris, 192 mM glycine, 20% methanol) for 12 to 14 hours at 60 volts (constant) and 4°C. The following primary antibodies were diluted in PBS containing 0.1% Tween (PBS-T) and 5% nonfat dry milk: pAb416 (Harlow et al., 1981) for detection of TAg expression, P5G6 (gift of D. Galloway) for detection of VP1 expression, and Ab8245 (Abcam) for detection of glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) expression as a loading control. Blots were washed in PBS-T, probed with horseradish peroxidase-conjugated anti-mouse IgG secondary antibody (Sigma), and developed using ECL+ reagent (Amersham) and exposure to film.

Fluorescent Focus Assay (FFA). Viral lysates were harvested at T = 0 (after one hour absorption at 37°C) and four days post-infection (or at T = 0 and 1, 2, 3, 4, and 6 days post-infection for the viral growth curve) by collecting and preparing infected cells and supernatants by three freeze/thaw cycles, as described above. Seventy percent confluent RPTE cells in 24-well plates were infected with 10-fold dilutions of viral lysates for four days at 37°C. Cells were fixed with 50% methanol/50% acetone for 10 min at room temperature, air dried for 15 min, wrapped in parafilm and stored overnight at -20°C, as an antigen retrieval step. Plates were thawed briefly at room temperature, rehydrated with PBS, and incubated for one hour at 37°C with pAb416 in PBS, followed by FITC-conjugated anti-mouse IgG secondary antibody (Sigma) in PBS with 0.005% Evans Blue stain. Titer was determined by counting five random fields in at least three replicate wells and is expressed as infectious units per ml (IU/ml). Statistical significance was determined using a two-tailed Student's t test assuming unequal variance and P values < 0.05 were considered significant.

RNA extraction and cDNA synthesis. Total cell RNA was harvested at two or four days post-infection using TRIzol reagent (Invitrogen) according to manufacturer's instructions. RNA samples were treated with DNaseI (Promega) to reduce contaminating DNA and the integrity of the RNA was confirmed by electrophoresis on an agarose gel. To generate cDNA, a reverse transcription reaction was performed using 1 μg RNA as template and the iScript cDNA Synthesis Kit (Bio-Rad), according to manufacturer's instructions.

Real Time PCR: Taqman Assay. Primers and probes were designed using Primer3 software (Rozen and Skaletsky, 2000) to amplify 90- and 105-base pair fragments of the

TAg and GAPDH genes, respectively; sequences are shown in Table 1. Primers were synthesized by Invitrogen and probes, tagged with 6-fluorescein (FAM) as the reporter dye at the 5' end and TAMRA-Sp as the quencher dye at the 3' end, were synthesized by Integrated DNA Technologies, Inc. (IDT). Reactions were performed in a total volume of 25 μl using TaqMan Universal PCR 2x master mix (Applied Biosystems), 2.5 μl cDNA template, 500 nM of each primer, and 200 nM probe. Amplification was performed in 96-well PCR plates (Bio-Rad) using the iCycler iQ5 Real Time Detection System (Bio-Rad) with the following PCR program: 2 min at 50°C; 10 min at 95°C; 40 cycles of denaturation at 95°C for 15 sec and annealing and extension at 56°C for 1 min. Results are presented as the fold change in TAg transcript levels, with the levels in samples treated with 250 U/ml IFN-γ arbitrarily set to one. Results were normalized to the levels of GAPDH transcripts present using the $2^{-\Delta\Delta C(T)}$ (Livak) method (Livak and Schmittgen, 2001). Statistical significance was determined using a two-tailed Student's t test assuming unequal variance and P values < 0.05 were considered significant.

Gene		Sequence (5' to 3')
TAg	Forward Primer	AAAAATGGAGCAGGATGTAAAGGT
	Reverse Primer	TCTTCTGTTCCATAGGTTGGCA
	Probe	AGCTACTCCAGGTTCCAAAATCAGGCTGA
GAPDH	Forward Primer	GCCTCAAGATCATCAGCAAT
	Reverse Primer	CTGTGGTCATGAGTCCTTCC
	Probe	AAGGTCATCCATGACAACTTTGGTATCG

Table 1. Sequences of primers and probes used in Taqman Real Time PCR assays.

Results

IFN-γ inhibits the expression of viral genes during infection with BKV. To begin our investigation of the role of cytokines and chemokines in regulating BKV infection, we examined the effect of several molecules on viral gene expression during infection of RPTE cells. The cytokines and chemokines used in this experiment were chosen for their well-known antiviral effects (IFN-α and IFN-γ) or because they are produced by proximal tubule epithelial cells following injury or stimulation (Daha and van Kooten, 2000). RPTE cells were infected with BKV, treated with IFN-γ, IFN-α, IL-6, IL-8, MCP-1, RANTES, or TNF-α, and total cell protein was harvested at four days post-infection. As a control, the ability of each cytokine to stimulate RPTE cells was analyzed by Western blot: phosphorylation of STAT1 was observed for IFN-γ and IFN-α, while phosphorylation of ERK1/2 was observed for IL-6, IL-8, MCP-1, and TNF-α; results for RANTES were inconclusive (data not shown). Western blot analysis of lysates probing for the viral proteins TAg, representing early gene expression, and VP1, representing late gene expression, revealed that only IFN-γ had a significant effect on viral gene expression (Figure 2.1A). Similar levels of GAPDH, a cellular housekeeping gene, were detected in all samples, showing that the inhibitory effect on viral gene expression was specific and could not be attributed to the known non-specific cellular effects of IFN-γ, namely the induction of anti-proliferative and pro-apoptotic pathways (Figure 2.1A). Comparable results were obtained when samples were normalized to the total number of cells per lysate (data not shown). Although IFN-α and IL-6 both appeared to have a slight inhibitory effect on TAg and VP1, the potential roles of these cytokines in regulating

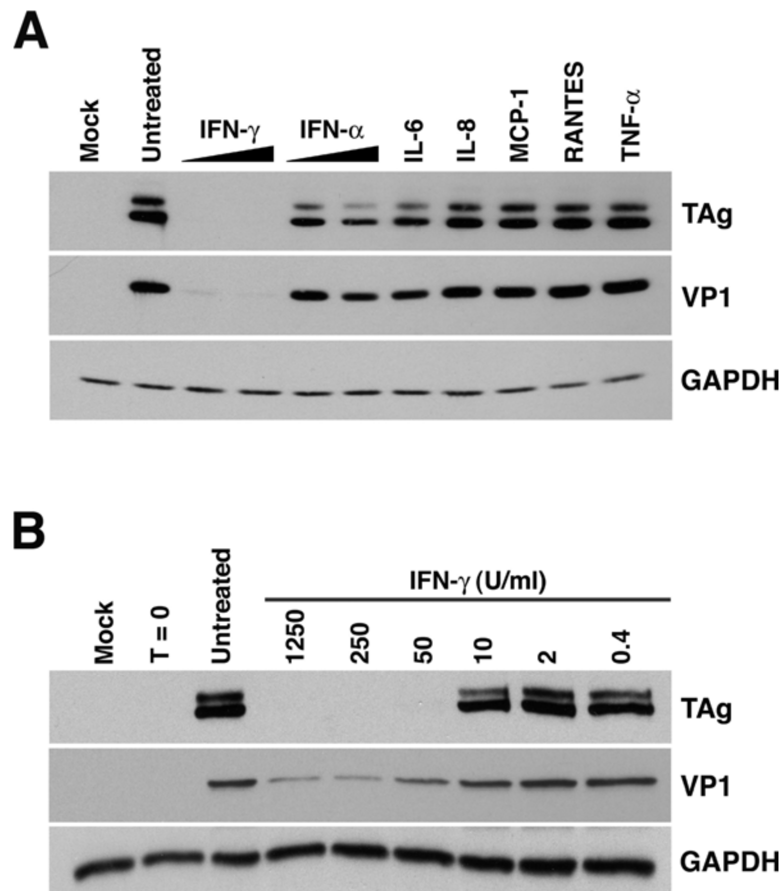

Figure 2.1. Dose-dependent IFN-γ inhibition of BKV gene expression. RPTE cells were infected with the TU strain of BKV at an MOI of 0.5 and treated with cytokines at three to six hours post-infection, and total cell protein was harvested at four days post-infection. Samples were analyzed by Western blotting, probing for TAg, VP1, and GAPDH. A) Infected cells were treated with 50 or 250 U/ml IFN-γ or IFN-α, 100 ng/ml IL-6, IL-8, MCP-1, or TNF-α, or 300 ng/ml RANTES. B) Infected cells were treated with the indicated concentrations of IFN-γ. Mock, Mock-infected samples with no cytokine treatment; untreated, BKV-infected samples with no cytokine treatment; T = 0, samples harvested directly after one hour of adsorption with BKV.

BKV replication was not pursued at this time. The dramatic inhibition of viral protein expression with IFN-γ treatment is the subject of further investigation in this study.

To determine whether IFN-γ exerts an inhibitory effect on BKV gene expression in a dose-dependent manner, RPTE cells were infected with BKV and treated with five-fold dilutions of IFN-γ. Total cell protein was harvested at four days post-infection and analyzed for TAg, VP1, and GAPDH expression by Western blotting (Figure 2.1B). At the three higher doses (1250, 250, and 50 U/ml IFN-γ), levels of TAg were undetectable while at the three lower doses (10, 2, and 0.4 U/ml IFN-γ), TAg expression approached the level of the untreated sample. At higher doses, VP1 expression was detectable but greatly reduced when compared to the untreated sample, and levels of VP1 increased as the dose of IFN-γ decreased. Samples harvested directly after the initial infection (one hour incubation with viral lysates), denoted T = 0, were analyzed to determine the amount of detectable VP1 due to input virions, which was negligible (Figure 2.1B). Thus, levels of VP1 in all samples corresponded to *de novo* protein expression during infection. Levels of GAPDH were similar for all samples, indicating equal protein loading and accounting for the general cellular effects of IFN-γ.

IFN-γ does not affect the kinetics of BKV replication. To determine whether IFN-γ-mediated inhibition of viral gene expression was the result of a delay in the progression of infection or a reduction in the level of gene expression, we infected RPTE cells with BKV, treated with 50 or 250 U/ml IFN-γ, and harvested total cell protein at 12 hour intervals over four days (Figure 2.2A). Samples were assayed for TAg, VP1, and GAPDH expression by Western blotting. In both untreated samples and samples treated with 50 U/ml IFN-γ, TAg expression was first detected at 36 hours post-infection, while

VP1 expression was first detected at 48 hours post-infection, indicating that there was no delay in the initiation of viral gene expression. Similar results were obtained from analysis of samples treated with 250 U/ml IFN-γ (data not shown). An interesting pattern of viral gene expression was apparent in treated samples: the levels of TAg and VP1 peaked at 72 and 84 hours post-infection, respectively, and then decreased at 96 hours post-infection (Figure 2.2A). This level of inhibition of viral gene expression was maintained up to 13 days post-infection (data not shown).

To examine the effect of IFN-γ on the timing and level of viral progeny produced, viral lysates were harvested at T = 0 and 1, 2, 3, 4, and 6 days post-infection from BKV-infected RPTE cells either untreated or treated with 50 or 250 U/ml IFN-γ. The titer of each lysate was determined by fluorescent focus assay, which detects the expression of TAg in newly infected cells (Figure 2.2B). The increase in viral titer of untreated and IFN-γ-treated samples was similar during the first 48 hours of infection, after which the untreated samples abruptly increased in viral titer whereas the IFN-γ-treated samples maintained the slower rate of increase seen in the first 48 hours. Interestingly, the viral titers of both untreated and IFN-γ-treated samples reached a plateau at four days post-infection; however, the titers of the IFN-γ-treated samples were approximately 16- and 20-fold lower (for 50 and 250 U/ml IFN-γ-treated samples, respectively) than the untreated samples at six days post-infection. The timing of viral protein expression and progeny production were similar for untreated samples and samples treated with 50 or 250 U/ml IFN-γ, and comparable results were obtained when cells were treated with IFN-γ prior to infection (data not shown). These data suggest that IFN-γ inhibits the level of viral gene expression and does not delay the progression of infection. This finding

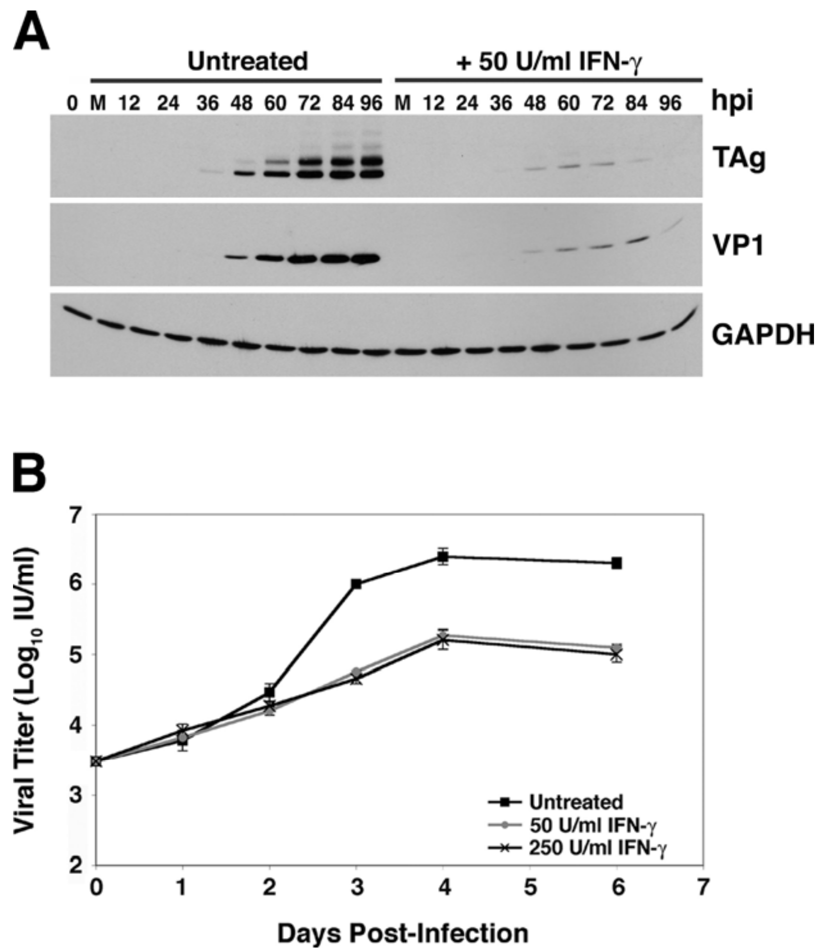

Figure 2.2. BKV replication kinetics in the presence of IFN-γ. RPTE cells were infected with the TU strain of BKV at an MOI of 0.5, treated with 50 (Figure 2.2A, 2.2B) or 250 U/ml (Figure 2.2B) IFN-γ at three to six hours post-infection. A) Total cell protein was harvested every 12 hours for four days. Samples were analyzed by Western blot, probing for TAg, VP1, and GAPDH. Untreated, BKV-infected samples with no IFN-γ treatment; M, mock-infected samples; hpi, hours post-infection; 0 hpi, samples harvested directly after one hour absorption with BKV (to detect protein from input virions). B) Viral lysates were harvested at 0, 1, 2, 3, 4, and 6 days post-infection and progeny production was determined by the fluorescent focus assay. Data are represented as the log of the viral titer in infectious units per ml (IU/ml) and samples were assayed in triplicate. Error bars are too small to be seen for some samples.

rules out a block at entry or trafficking to the nucleus as the mechanism of IFN-γ inhibition.

Treatment with IFN-γ results in a reduction in the level of early region transcripts. The previous experiments demonstrated the inhibitory effect of IFN-γ on the level of viral protein expression and progeny production. To determine whether IFN-γ-mediated inhibition occurs at the level of transcription or translation, we examined the effect of IFN-γ on viral transcript production. Total cell RNA was harvested at 48 or 96 hours post-infection from RPTE cells infected with BKV and treated with 50 or 250 U/ml IFN-γ. RNA samples were analyzed by real time reverse-transcription PCR (real time RT-PCR) to detect the levels of TAg transcripts. By designing the TAg primers and probe set to amplify a 90-base pair amplicon across the splice site of the early region, amplification was limited to the amplicon produced from the TAg cDNA template, as opposed to that from the tAg or unspliced cDNA template. Results were normalized to the levels of GAPDH transcripts to account for the non-specific cellular effects of IFN-γ, using the $2^{-\Delta\Delta C(T)}$ method and are presented as the fold change in TAg transcript levels, with the levels in samples treated with 250 U/ml IFN-γ arbitrarily set to one.

At 48 hours post-infection, there was a modest 1.6-fold decrease in TAg transcript levels with 50 and 250 U/ml IFN-γ treatment (Figure 2.3A). These results were confirmed by Northern blot analysis, using a probe specific for the entire BKV early region to detect levels of all species of early region transcripts (data not shown). A similar level of inhibition was observed at 48 hours post-infection at the level of protein expression (Figure 2.2A). At 96 hours post-infection, there was a more dramatic and highly significant inhibitory effect with IFN-γ treatment, such that treatments with 50 and

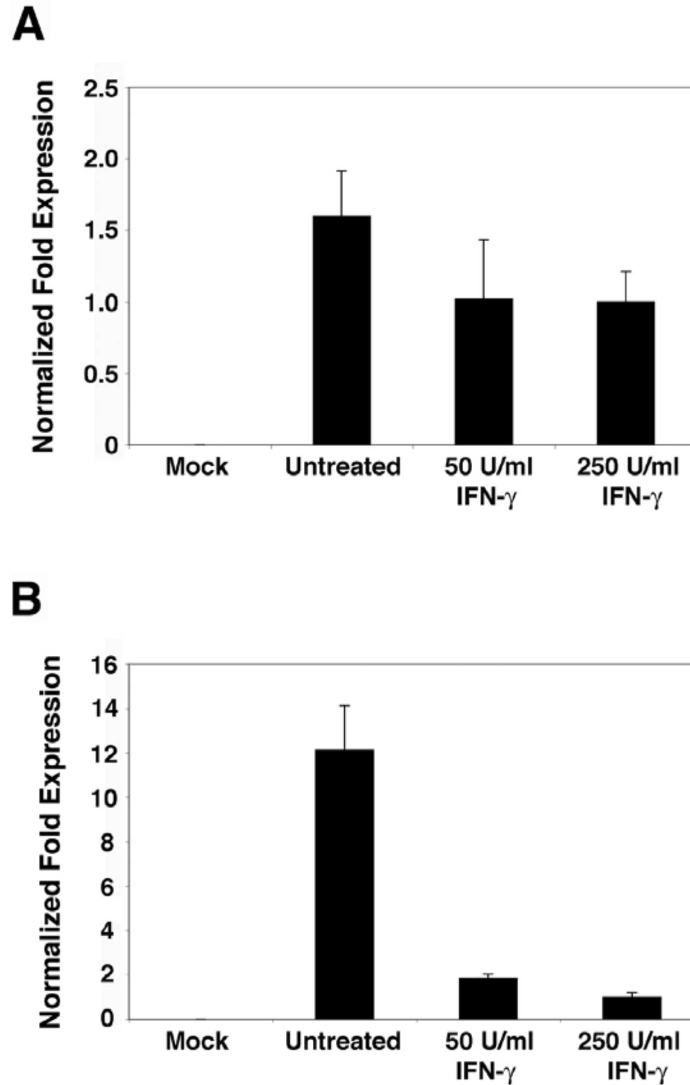

Figure 2.3. Effect of IFN-γ on viral early region transcript levels. RPTE cells were infected with the TU strain of BKV at an MOI of 0.5, treated with 50 or 250 U/ml IFN-γ at three to six hours post-infection, and total cell RNA was prepared at (A) 48 or (B) 96 hours post-infection. The level of TAg transcripts in each sample was determined using real time RT-PCR, normalizing to level of GAPDH transcripts; samples treated with 250 U/ml IFN-γ were arbitrarily set to one. Each bar represents the average of two (Figure 2.3A) or three (Figure 2.3B) independent experiments analyzed in triplicate in the same assay. Mock, mock-infected samples with no IFN-γ treatment; untreated, BKV-infected samples with no IFN-γ treatment.

250 U/ml IFN-γ resulted in 6.6-fold ($P < 0.005$) and 12.1-fold ($P < 0.004$) reductions in TAg transcript levels, respectively (Figure 2.3B). These findings are consistent with our previous observations of the protein levels at 96 hours post-infection (Figure 2.2A). The relative correlation of real time RT-PCR data (measuring the effect of IFN-γ on the steady-state level of TAg mRNA) and observations made from Western blot analysis (showing the effect of IFN-γ on the level of viral protein expression) suggest predominately IFN-γ-mediated effects on transcription. However, IFN-γ may also mediate an inhibitory effect at the level of translation. Future studies on the mechanism of inhibition will provide more insight on this subject.

Effect of IFN-γ during infections with different MOIs. To investigate whether the inhibitory effect of IFN-γ is dependent on levels of input virus, RPTE cells were infected with five-fold dilutions of BKV. These infections of varying MOIs may represent different stages of BKV infection in the host. For example, an infection at an MOI of 0.1 might be similar to the subclinical state of persistence seen in immunocompetent hosts, while an infection at an MOI of 12.5 might be more similar to the reactivation and lytic infection of BKV preceding the development of PVN in immunosuppressed hosts. Infected cells were treated with 50 or 250 U/ml IFN-γ and total cell protein was harvested at two, three, and four days post-infection for analysis of viral protein expression by Western blotting (Figure 2.4A, 2.4B). Regardless of the MOI used during the infection, TAg and VP1 expression were strongly inhibited with IFN-γ treatment and peak protein levels occurred at three days post-infection, similar to the pattern seen in previous experiments. Not surprisingly, levels of TAg and VP1 expression were higher in samples from infections with more input virus, but IFN-γ treatment still mediated a strong

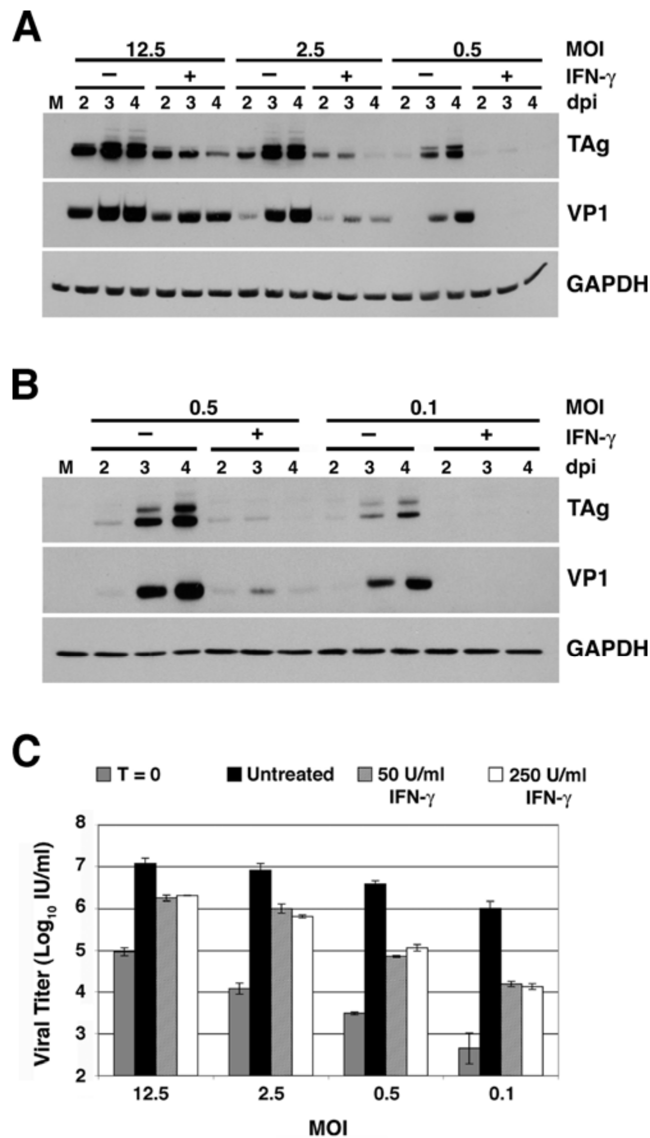

Figure 2.4. Effect of IFN-γ during infections with different MOIs. RPTE cells were infected with the TU strain of BKV at MOIs of 12.5, 2.5, 0.5, or 0.1 and treated with 50 U/ml (Figure 2.4A, 2.4B, 2.4C) or 250 U/ml (Figure 2.4C) IFN-γ at three to six hours post-infection. A and B) Total cell protein was harvested at two, three, and four days post-infection and analyzed by Western blot, probing for TAg, VP1, and GAPDH. The analysis of total cell protein harvested from infection at an MOI of 0.5 was repeated in Figure 2.4B for direct comparison to samples from infection at an MOI of 0.1. M, mock-infected samples with no IFN-γ treatment; dpi, days post-infection. C) Viral lysates were harvested at T = 0 (after one hour absorption with BKV) and four days post-infection and viral progeny production was determined by the fluorescent focus assay. Data are represented as the log of the viral titer in infectious units per ml (IU/ml) and samples were assayed in triplicate. Untreated, BKV-infected samples with no IFN-γ treatment.

reduction in viral gene expression compared to untreated samples with the same infection conditions. These data suggest that IFN-γ-mediated inhibition may be important for controlling BKV replication in both immunocompetent and immunosuppressed hosts.

Viral lysates were harvested at four days post-infection from cells infected in these same conditions and analyzed for viral progeny production using the fluorescent focus assay. While significant inhibitory effects mediated by IFN-γ were seen in all treated samples (for MOIs of 12.5, 2.5, 0.5, $P < 0.05$; for MOI of 0.1, $P = 0.05$), the effect was greater when less input virus was used during the infection (Figure 2.4C). Cells treated with IFN-γ and infected at an MOI of 0.1 showed an almost 80-fold reduction in viral progeny production, while cells infected at an MOI of 12.5 (7-fold reduction), 2.5 (13-fold reduction), and 0.5 (53-fold reduction) were affected less severely by IFN-γ treatment. A comparison of viral titers of IFN-γ-treated samples with those of input virus (T = 0) showed that BKV progeny were produced in the presence of IFN-γ. This suggests that, despite the inhibitory effects observed, IFN-γ is not driving BKV infection into a state of latency, in which the complete absence of late gene expression and progeny production would be expected.

Response of various BKV strains to IFN-γ treatment. To determine whether the inhibitory effect of IFN-γ on viral replication and gene expression is consistent for different strains of BKV, we infected RPTE cells with the TU strain, which was used throughout the above experiments, and two additional strains, Dunlop and Proto-2. Cells were treated with 50 or 250 U/ml IFN-γ and total cell protein was harvested at two, three, and four days post-infection for analysis of viral protein expression by Western blotting (Figure 2.5A). For each strain, TAg and VP1 expression were strongly inhibited by

treatment with 50 U/ml IFN-γ and as before, viral protein levels in treated samples peaked at three days post-infection (Figure 2.5A). Results were similar for samples treated with 250 U/ml IFN-γ (data not shown). These data suggest that all strains of BKV may respond similarly to IFN-γ-mediated inhibition of viral gene expression. In addition, viral lysates were harvested at the initiation of infection (T = 0) and four days post-infection from RPTE cells infected with the three BKV strains and treated with 50 or 250 U/ml IFN-γ (Figure 2.5B). IFN-γ treatment significantly inhibited progeny production for each of the strains ($P < 0.05$): viral titer was reduced in the presence of IFN-γ by as much as 456-fold for infection with the Dunlop strain, 114-fold for infection with the Proto-2 strain, and 53-fold for infection with the TU strain (Figure 2.5B).

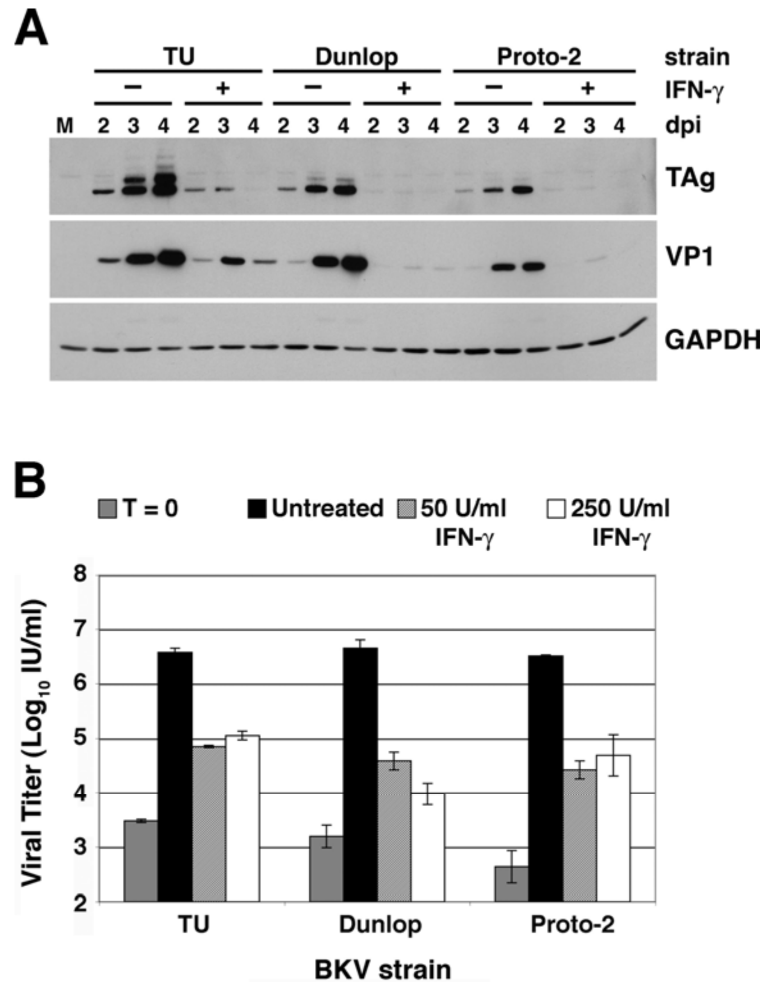

Figure 2.5. Response of various BKV strains to IFN-γ treatment. RPTE cells were infected with the TU, Dunlop, or Proto-2 strains of BKV at an MOI of 0.5 and treated with 50 U/ml (Figure 2.5A, 2.5B) or 250 U/ml (Figure 2.5B) IFN-γ at three to six hours post-infection. A) Total cell protein was harvested at two, three, and four days post-infection and analyzed by Western blot, probing for TAg, VP1, and GAPDH. M, mock-infected samples with no IFN-γ treatment; dpi, days post-infection. B) Viral lysates were harvested at T = 0 (after one hour absorption with BKV) and four days post-infection and viral progeny production was determined by the fluorescent focus assay. Data are represented as the log of the viral titer in infectious units per ml (IU/ml) and samples were assayed in triplicate. Untreated, BKV-infected samples with no IFN-γ treatment.

Discussion

Polyomavirus nephropathy is a complication associated with kidney transplantation resulting from the reactivation of BKV. Our knowledge about the immune response to BKV is limited, making the management of PVN difficult. The experiments described in this paper begin to characterize the inhibitory effect of IFN-γ on the lytic infection of proximal tubule epithelial cells by BKV. We have shown that IFN-γ specifically inhibits viral gene expression and progeny production in a dose-dependent manner. In addition, infected cells exposed to IFN-γ have lower levels of TAg transcripts than untreated cells, suggesting that IFN-γ-mediated inhibition occurs at the level of transcription. Cells infected with different MOIs of BKV responded similarly to treatment with IFN-γ; however the more virus present, the weaker the inhibitory effect. In addition, IFN-γ had a significant inhibitory effect on viral gene expression and progeny production during infections with three different strains of BKV. These findings suggest that IFN-γ plays an important role in regulating BKV infection.

It is important to note that samples treated with IFN-γ maintained detectable *de novo* viral gene expression and progeny production. It is unclear whether this is a result of a low level of viral replication in all cells due to incomplete inhibition by IFN-γ, or a normal level of replication in a fraction of cells that were unresponsive to IFN-γ. In the latter scenario, IFN-γ may force BKV into a state of latency in responsive cells. However, the finding that viral replication continued to some extent in the presence of IFN-γ correlates well with the observation that healthy immunocompetent individuals shed BKV in their urine periodically throughout their lives. A reduction of IFN-γ

production by antigen-specific T lymphocytes in immunosuppressed patients may be, in part, responsible for reactivation and subsequent development of BKV-associated disease.

An interesting pattern of viral gene expression was noted in samples exposed to IFN-γ: protein levels, though severely reduced when compared to untreated samples, peaked at three days post-infection and then declined until a steady, low level of expression was reached. We hypothesize that treatment with IFN-γ may either shorten the duration of TAg expression or force the virus into a persistent or latent state. Since we observed progeny production at times when TAg expression was either very low or undetectable by Western blot, a minimal threshold level of TAg seems to be sufficient to facilitate the progression of a productive BKV infection.

Activation of the IFN-γ signaling cascade as a result of IFN-γ binding to the surface receptor has a multitude of effects on the cell (Schroder et al., 2004; Sen, 2001). IFN-γ has overall anti-proliferative and pro-apoptotic effects, mediated primarily by protein kinase R and IRF-1, respectively. In our studies, we have accounted for these non-specific cellular effects by normalizing each sample to the levels of a housekeeping gene, GAPDH or to cell number. IFN-γ signaling also plays a major role in activating and recruiting cells of the immune system, for example, by up-regulating the surface expression of MHC class I and II molecules and inducing expression of inflammatory cytokines. However, the system we used to study the lytic infection of BKV does not incorporate cells of the immune system and thus eliminates immune activation from the potential mechanisms of IFN-γ-specific inhibition of BKV replication. In addition, IFN-γ signaling activates the type I IFN cascade, which establishes a general antiviral

environment within the cell. However, the direct treatment of infected cells with IFN-α has little effect on viral gene expression (Figure 2.1A). The last major category of IFN-γ signaling effects involves the activation or repression of transcription factors that regulate the expression of genes. We speculate that the mechanism by which IFN-γ inhibits BKV replication employs regulatory transcription factors that act on the early and late viral promoters. However, regulation of the early promoter to inhibit TAg expression alone would be sufficient to explain a decrease in levels of TAg, VP1, and viral progeny production, since TAg is a key mediator of the progression of the BKV life cycle. IFN-γ-activated transcription factors include members of the STAT and IRF family, ISGF3, c-Myc, and c-Jun, as well as other factors affected by downstream elements of the IFN-γ signaling cascade and subsequently regulated at a later time (de Veer et al., 2001; Der et al., 1998). We are currently investigating candidate IFN-γ-regulated factors that may be responsible for the inhibitory effects on BKV.

The non-coding control region (NCCR) is the region of greatest variability between different strains of BKV. Rearrangements of the transcription factor binding blocks (designated O-P-Q-R-S in the archetypal BKV strain WW) occur frequently in tissue culture systems and seem to arise in individuals with high viral loads or reactivation. We examined the responses of three different strains of BKV to IFN-γ treatment during lytic infection. BKV TU (NCCR structure: O-P-Q-R_{1-12}-P_{16-68}-Q_{1-35}-R_{52-63}-S) and BKV Dunlop (O-P-$P_{1-7;26-68}$-P_{1-64}-S) are strains commonly found in immunocompetent individuals, while BKV Proto-2 (O-P-$P_{1-7;26-68}$-P-Q_{1-28}-S_{7-63}) was isolated from the urine of HIV-infected patients (Doerries et al., 1994; Moens and Rekvig, 2001; Sundsfjord et al., 1994; Sundsfjord et al., 1990). The observation that

IFN-γ is able to inhibit viral gene expression and progeny production of three different strains of BKV with dissimilar NCCR structures suggests that our findings may be applicable to all strains of BKV. Therefore, we speculate that transcription factor binding sites common to all three NCCRs (and potentially to most BKV strains) mediate the inhibitory effect of IFN-γ on the viral promoters.

It is still not understood what factors cause certain kidney transplant patients to undergo reactivation of BKV and develop PVN, while others on the same immunosuppressive regimen do not. One possibility involves differences in host genetics. For example, genetic polymorphisms of the *IFNG* gene may render some patients more susceptible to BKV reactivation than others. Polymorphisms are frequent in the promoter region of the *IFNG* gene and affect the level of IFN-γ normally produced in the body (Pravica et al., 1999; Pravica et al., 2000). Recent reports attempt to establish a correlation between certain *IFNG* polymorphisms and susceptibility to various diseases, including HPV-induced cervical cancer (Lai et al., 2005), tuberculosis (Lopez-Maderuelo et al., 2003), and parvovirus B19 infections (Kerr et al., 2003). It is possible that kidney transplant patients with *IFNG* polymorphisms resulting in lower levels of IFN-γ production are naturally more susceptible to reactivation of BKV under immunosuppressive therapies. Screening of patients for such polymorphisms may help to determine the appropriate level of immunosuppression required to prevent graft rejection but still maintain the ability of the immune system to repress BKV reactivation. In addition, treatment with IFN-γ may prove beneficial as an alternative to reducing immunosuppressive therapies upon detection of active BKV replication. There are precedents for successful interferon therapy: IFN-α has been used to treat a wide-range of

cancers, hepatitis B virus, and hepatitis C virus, while IFN-γ has proven effective against chronic granulomatous disease (Pestka et al., 2004).

In conclusion, IFN-γ has a potent inhibitory effect on BKV gene expression, both at the level of transcription and translation, and viral progeny production in proximal tubule cells. This effect is similar for the three different strains of BKV examined and is more effective at inhibiting gene expression and progeny production in the presence of less virus. The exact mechanism of IFN-γ-mediated inhibition is not yet known, but we speculate that the activity of the BKV promoters is regulated by IFN-γ responsive transcription factors. These findings expand the characterization of the host immune response to BKV and may lead to new approaches for the prevention of BKV reactivation in kidney transplant recipients.

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CHAPTER III

TRANSFORMING GROWTH FACTOR-BETA-MEDIATED REGULATION OF BK VIRUS GENE EXPRESSION

BK virus (BKV) is a human polyomavirus that has a well-established role in complications following transplantation and immunosuppression (Comoli et al., 2006; Nickeleit and Mihatsch, 2006). In particular, BKV is the causative agent of polyomavirus nephropathy (PVN) in up to 10% of kidney transplant recipients, resulting in loss of graft function in 10-80% of those affected (Hirsch et al., 2006). BKV reactivation is also associated with hemorrhagic cystitis (HC) in bone marrow transplant recipients, with approximately 50% of patients with active BKV viruria progressing to HC (Bedi et al., 1995; Pavlakis et al., 2006). Despite extensive investigation, there has been limited success in identifying antiviral treatments for BKV reactivation and lytic infection (Josephson et al., 2006; Trofe et al., 2006). Typically, upon diagnosis of BKV reactivation and PVN, the immunosuppressive regimen of the patient is reduced to allow the immune system to fight the viral infection. This approach, however, increases the risk of graft rejection. Thus it is important to investigate the immune response to BKV to identify immune components that control viral replication.

BKV was first isolated from the urine of a kidney transplant patient with ureteral stenosis in 1971 (Gardner et al., 1971). Primary infection with BKV occurs early in childhood, with seroconversion occurring by the age of 10 in up to 80% of the human population (Knowles, 2006). Following the primary infection, BKV undergoes viremic dissemination and establishes a lifelong persistent infection primarily in the cells of the kidney and urinary tract (Chesters et al., 1983; Heritage et al., 1981). More specifically, tubular epithelial cells of the kidney and epithelial cells of the urinary tract are major sites of BKV persistence and reactivation (Doerries, 2006; Nickeleit and Mihatsch, 2006).

BKV has a non-enveloped, icosahedral virion composed of three proteins, VP1, VP2, and VP3, which encapsidate a circular double-stranded DNA genome of approximately 5.2 kb. The genome can be divided into three distinct regions: the early region, which contains the coding sequences for large tumor antigen (TAg) and small tumor antigen (tAg); the late region, which contains the coding sequences for VP1, VP2, VP3, and agnoprotein; and the non-coding control region (NCCR), which contains the origin of replication and the viral early and late promoters. The NCCR is used to distinguish one strain of BKV from another because of the propensity of this region to acquire point mutations and structural rearrangements (Moens and Van Ghelue, 2005). BKV strains can be divided into two classes: archetypal (pre-rearranged) strains, presumed to be the infectious and transmissible virus, and rearranged strains, which are predominately isolated from tissue biopsy samples (Cubitt, 2006). The NCCRs of archetypal strains are structurally divided into blocks of transcription factor binding sites, arbitrarily designated O (142 bp, containing the origin of replication, TATA box, and TAg binding sites), P (68 bp), Q (39 bp), R (63 bp), and S (63 bp) (Markowitz and

Dynan, 1988; Moens et al., 1995). Rearranged BKV strains result from the restructuring of the archetypal NCCR, such that certain blocks (primarily P, Q, and R) are, in whole or in part, duplicated or deleted (Moens and Van Ghelue, 2005).

It is commonly reported that the predominant NCCR configuration actively shed in urine is archetypal (Markowitz et al., 1991; Negrini et al., 1991; Sharma et al., 2007; Sundsfjord et al., 1999; Takasaka et al., 2004), and that NCCR rearrangements seem to be isolated more frequently from the tissues and sera of patients with high viral loads (Boldorini et al., 2001; Gosert et al., 2008; Stoner et al., 2002). In addition, changes in the NCCR structure arise spontaneously in tissue culture and these rearrangements enhance the ability of the virus to replicate and transform cells (Rubinstein et al., 1991; Watanabe and Yoshiike, 1985; Watanabe and Yoshiike, 1986). It is possible that changes in the viral promoter region can also result in altered pathogenesis, such as a heightened ability to reactivate or disseminate. Furthermore, rearrangements may affect the cell tropism of BKV, allowing infection of other cell types in addition to kidney and urinary epithelial cells. The confounding observation, however, is that as of yet there is no apparent correlation between BKV NCCR structure and clinical outcome (Sharma et al., 2007).

TGF-β is a secreted cytokine having three isoforms in mammals (TGF-β1, TGF-β2, TGF-β3), all with similar functions involved in the regulation of cell proliferation, differentiation, and immune suppression (Feng and Derynck, 2005; Li et al., 2006). TGF-β is produced by many different cell types, including renal epithelial cells, a major site of BKV reactivation. Interestingly, the expression of TGF-β is enhanced in the presence of immunosuppressive therapies commonly administered to renal transplant

patients (Khanna et al., 1999a; Khanna et al., 1999b; McMorrow et al., 2005; Shihab et al., 1996). We hypothesized that TGF-β would have an upregulatory effect on BKV gene expression and replication, correlating with the evident reactivation of BKV in kidney transplant recipients.

TGF-β is initially expressed as an inactive complex of precursor polypeptides that undergoes activation by proteolytic cleavage. Upon maturation, TGF-β can bind to TGF-β receptor II dimers, resulting in the recruitment and phosphorylation of TGF-β receptor I dimers (Shi and Massague, 2003). Once activated, TGF-β receptor I recruits and phosphorylates Smad2 and Smad3 proteins, the primary components of the TGF-β signaling cascade (Massague et al., 2005). Phosphorylated Smad2 and Smad3 proteins can then form a complex with Smad4, resulting in the nuclear translocation of these proteins. This complex has some weak intrinsic DNA binding activity of its own, but is more effective in regulating TGF-β-dependent gene expression in conjunction with cellular transcriptional co-activators that have high DNA binding activity and specificity (Brown et al., 2007; Massague and Wotton, 2000; Shi et al., 1998).

In this report, we characterize the effect of TGF-β on the lytic infection of BKV in renal proximal tubule epithelial (RPTE) cells and on the activity of the viral early region promoter. We demonstrate that the response to TGF-β-mediated regulation is dependent on the strain of BKV and thus the NCCR structure. We show that upregulation by TGF-β maps to a short region of the promoter that most likely contains two distinct transcription factor binding sites. These findings demonstrate transcriptional regulation of BKV by a cytokine that is found at elevated levels in transplant patients.

Materials and Methods

Cell culture and reagents. Primary human renal proximal tubule epithelial cells (RPTE cells, Cambrex) were maintained in renal epithelial cell basal medium (REBM, Cambrex) supplemented with human epidermal growth factor, fetal bovine serum (FBS), hydrocortisone, epinephrine, insulin, triiodothyronine, transferrin, and GA-1000 as indicated for renal epithelial cell growth medium (REGM, supplements obtained as REGM SingleQuots, Cambrex). HT-1080 cells (ATCC CCL-121) were maintained in Dulbecco's Modified Eagle Medium (Gibco) containing 10% FBS (Cambrex), 100 units/ml penicillin, and 100 μg/ml streptomycin (Cambrex). Both RPTE and HT-1080 cells were grown at 37°C with 5% CO_2 in a humidified incubator. Recombinant human TGF-β1, produced in A293 cells (Peprotech, Inc.), was reconstituted according to manufacturer's recommendations and used at a concentration of 10 ng/ml.

Viruses. BKV stocks were prepared from genomic clones of TU (cloned into the EcoRI site of pGEM-7Zf(-)), and Dunlop and Proto-2 (cloned into the BamHI site of pBR322, gift of Peter Howley), as previously described (Abend et al., 2007). The resulting crude viral stocks were titrated by fluorescent focus assay as previously described (Abend et al., 2007) and the integrity of the NCCR was confirmed by sequencing of PCR products.

Infections. RPTE cells at 70% confluence were infected with the TU, Dunlop, or Proto-2 strains of BKV in REGM at an MOI of 0.5 IU/cell (infectious units per cell), incubating for one hour at 37°C. Viral lysates used for the infection were replaced with fresh REGM. TGF-β was added at three to four hours post-infection (hpi).

Western blotting. Total cell protein was harvested at three days post-infection (dpi) using E1A lysis buffer (Harlow et al., 1986) supplemented with 5 μg/ml PMSF, 5 μg/ml

aprotinin, 5 μg/ml leupeptin, 0.05 M sodium fluoride, and 0.2 mM sodium orthovanadate. The Bio-Rad protein assay was used to determine the protein concentration of each lysate and 10 μg of protein were electrophoresed on an 4-20% Tris-glycine polyacrylamide gel (Lonza) and analyzed by Western blotting for the expression of viral early protein TAg and GAPDH as previously described (Abend et al., 2007).

RNA extraction and cDNA synthesis. Total cell RNA was harvested at 24, 36, 48, 72, and 96 hpi using TRIzol reagent (Invitrogen) according to manufacturer's instructions. Samples were treated with DNase I (Promega) to reduce contaminating DNA, and RNA integrity was confirmed by electrophoresis on an agarose gel. To generate cDNA, reverse transcription reactions were performed on 1 μg of input RNA using the iScript cDNA Synthesis Kit (Bio-Rad), according to manufacturer's instructions.

Real-Time PCR: TaqMan Assay. Primers and probes used to assay TAg and GAPDH transcript levels are reported previously (Abend et al., 2007). PCR reactions were performed in a total volume of 25 μl using TaqMan Universal PCR 2x master mix (Applied Biosystems), 2.5 μl cDNA template, 500 nM of each primer, and 200 nM probe. The iCycler iQ5 Real-Time Detection System (Bio-Rad) was used for amplification with the following PCR program: 2 min at 50°C; 10 min at 95°C; 40 cycles of denaturation at 95°C for 15 sec and annealing and extension at 56°C for 1 min. Results are presented as the fold change in TAg transcript levels, with the relative level observed at 24 hpi, untreated, arbitrarily set to one. Results are normalized to the levels of GAPDH transcripts present using the $2^{-\Delta\Delta C(T)}$ (Livak) method (Livak and Schmittgen, 2001).

Generation of luciferase constructs. The NCCRs of four strains of BKV were amplified from their genomic clones using the primers

Agno1 (5' AGTGCTAGCGCCTTTGTCCAGTTTAACT 3') and

LTAg2 (5' AGTCTCGAGAAATAGTTTTGCTAGGCCTCA 3'), which contain the restriction endonuclease sites for NheI and XhoI, respectively (underlined). Polymerase chain reactions utilizing these primers produced 350-400 bp fragments spanning the NCCR from the start codon of agnoprotein to 35 bp before the TAg start codon. These fragments were first cloned into the pGEM-T Easy vector (Promega) and then subcloned into the luciferase vector pGL2-basic (Promega) by means of the NheI and XhoI sites. In these resulting luciferase constructs (pGL2-TU, pGL2-Dik, pGL2-Dunlop, pGL2-Proto-2), the BKV early promoter drives the expression of the firefly luciferase gene.

Site-directed mutagenesis. The following primers were synthesized and HPLC purified to introduce a point mutation (underlined) at nucleotide 362 (GenBank accession no. **DQ305492**) in the BKV TU NCCR: TU-SmtFOR (5' TCGCAAAACATGT CTGTGTGGCTGCTTTCCGG 3'), TU-SmtREV (5' CCGGAAAGCA GCCACACAGACATGTTTTGCGA 3'). The following primers were synthesized and HPLC purified to insert 6 bp normally present only in the TU NCCR into the BKV Dik NCCR (underlined): Dik+6TUFOR (5' AAACATGTCTGTCTGGCTGC TTTCCGGTTTCACTCCTTTGG 3'), Dik+6TUREV (5' CCAAAGGAGTGAAACCG GAAAGCAGCCAGACAGACATGTTT 3'). Mutagenesis was performed following the protocol for the QuikChange II Site-Directed Mutagenesis Kit (Stratagene) using the primer pairs at 1.25 nM each, 100 ng of pGL2-TU or pGL2-Dik as template, 1 mM dNTPs, and 1.25 U Native *Pfu* DNA Polymerase (Stratagene) in 25 µl total reaction volume. The following two-step PCR program was used: 3 min at 95°C; 18 cycles of denaturation at 95°C for 15 sec and annealing and extension at 68°C for 12 min.

Luciferase assays. RPTE or HT-1080 cells were grown in 12-well tissue culture-treated plates to 60% confluence. Firefly luciferase constructs (pGL2-*BKV strain*) and a promoterless control *Renilla* luciferase plasmid (pRL-Null, Promega) were cotransfected into RPTE cells at a ratio of 9:1, with a total of 0.6 μg of DNA per well, using the Effectene Transfection reagent (Qiagen) according to manufacturer's recommendations. TGF-β was added at three to four hours post-transfection (hpt) and total cell lysates were harvested at 48 hpt in 1x Passive Lysis Buffer (Promega). Luciferase assays were performed on triplicate samples using the Dual-Luciferase Reporter Assay (Promega), according to manufacturer's recommendations. Results are expressed as relative light units (RLU) of firefly luciferase activity normalized to RLU of *Renilla* luciferase activity. TGF-β did not significantly affect the levels of *Renilla* luciferase activity (data not shown). Statistical significance was determined using a two-tailed Student's *t* test assuming unequal variance, and P values < 0.01 were considered significant.

Results and Discussion

TGF-β-mediated regulation of BKV gene expression during infection. We first investigated the regulatory potential of TGF-β on BKV gene expression during lytic infection of RPTE cells. We examined the levels of TAg at three dpi in cells infected with three strains of BKV that differ significantly in their NCCR structures: TU (O-P-Q-$R_{1\text{-}12}$-$P_{16\text{-}68}$-$Q_{1\text{-}35}$-$R_{52\text{-}63}$-S), Dunlop (O-P-$P_{1\text{-}7;26\text{-}68}$-$P_{1\text{-}64}$-S), and Proto-2 (O-P-$P_{1\text{-}7;26\text{-}68}$-P-$Q_{1\text{-}28}$-$S_{7\text{-}63}$). TGF-β only significantly affected the expression of viral proteins in cells infected with the TU strain of BKV, as shown by the upregulation of TAg levels (Figure 3.1A). TAg expression remained relatively unchanged in cells infected with the Dunlop and Proto-2 strains. To demonstrate that the effect was specific to viral gene expression and not a result of TGF-β-mediated inhibition of epithelial cell proliferation, samples were also analyzed for levels of GAPDH, a cellular housekeeping gene. These results indicate that the presence of TGF-β affects BKV gene expression in a strain-specific manner.

To determine whether TGF-β-mediated regulation occurs during transcription or translation, we examined the levels of early gene transcripts during the course of BKV TU infection. Total cell RNA was harvested at 24, 36, 48, 72, and 96 hpi and analyzed by a real-time RT-PCR assay to detect TAg transcripts in the absence or presence of TGF-β (Figure 3.1B). Results were normalized to the levels of GAPDH mRNA present in the samples. TGF-β had a prominent effect on TAg transcription at early time points, with 2.5-fold upregulation in transcript levels at 24 and 36 hpi, and 3.3-fold upregulation over untreated cells at 48 hpi. At the later stages of infection the effect of TGF-β was less pronounced, with only 1.8-fold upregulation in TAg transcripts at both 72 and 96

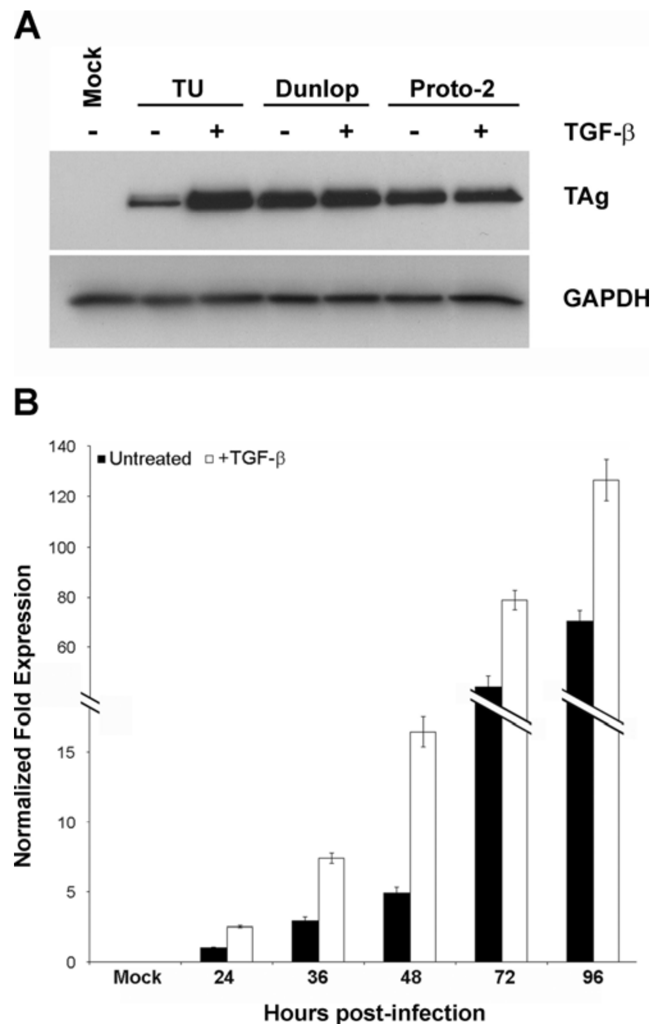

Figure 3.1. TGF-β upregulates BKV TU gene expression during infection. A) RPTE cells were infected with the indicated strains of BKV at an MOI of 0.5 IU/cell and treated with 10 ng/ml TGF-β at three to four hpi. Total cell protein was harvested at three dpi and analyzed by Western blot, probing for TAg and GAPDH. Mock, mock-infected samples with no TGF-β treatment. B) RPTE cells were infected with the TU strain of BKV at an MOI of 0.5 IU/cell in the presence or absence of 10 ng/ml TGF-β, and total cell RNA was prepared at 24, 36, 48, 72, and 96 hpi. Relative TAg transcript levels were determined using real time RT-PCR, normalizing to levels of GAPDH transcripts in each sample. One representative experiment is shown; triplicate samples were analyzed in the same assay. Fold expression of TAg at 24 hpi, untreated was arbitrarily set to one. Mock, mock-infected samples with no TGF-β treatment.

hpi. These results suggest that TGF-β-mediated regulation occurs at the level of transcription. Furthermore, the timing and limited duration of the upregulation suggest that transcription factors involved in the TGF-β signaling cascade are responsible for the effect.

BKV early promoter activity in the presence of TGF-β. We next set out to examine the effect of TGF-β on the viral early promoter. We cloned the NCCRs of four BKV strains [TU, Dunlop, Proto-2, Dik (archetypal)] into a luciferase reporter plasmid, such that the viral early promoter drives the expression of the firefly luciferase gene. This assay permitted us to examine the effect of TGF-β on promoter activity in the absence of viral gene expression and other BKV genomic sequences. RPTE cells were cotransfected with the BKV promoter-driven luciferase plasmids and a promoterless control *Renilla* luciferase plasmid in the absence or presence of TGF-β, and lysates were assayed for luciferase activity at 48 hpt (Figure 3.2A). The results confirmed that only the TU early promoter was affected by TGF-β treatment, while the other three viral promoters showed no significant change in activity in the presence of TGF-β (1.4-, 1.1-, and 1.1-fold change for Dik, Dunlop, and Proto-2 promoters, respectively). The 2.5-fold upregulation ($P = 0.009$) in TU promoter activity was similar to that seen during BKV infection of RPTE cells (Figure 3.1). The results of this assay suggest that the effect of TGF-β is mediated solely at the promoter and is therefore driven by specific transcription factors.

The regulation of BKV early promoter activity by TGF-β signaling may also depend on the cell type examined. The luciferase assay described above was also performed in HT-1080 cells, a human fibrosarcoma cell line (Figure 3.2B). The TU and Dik promoters responded similarly to TGF-β treatment in both cell types: TU promoter

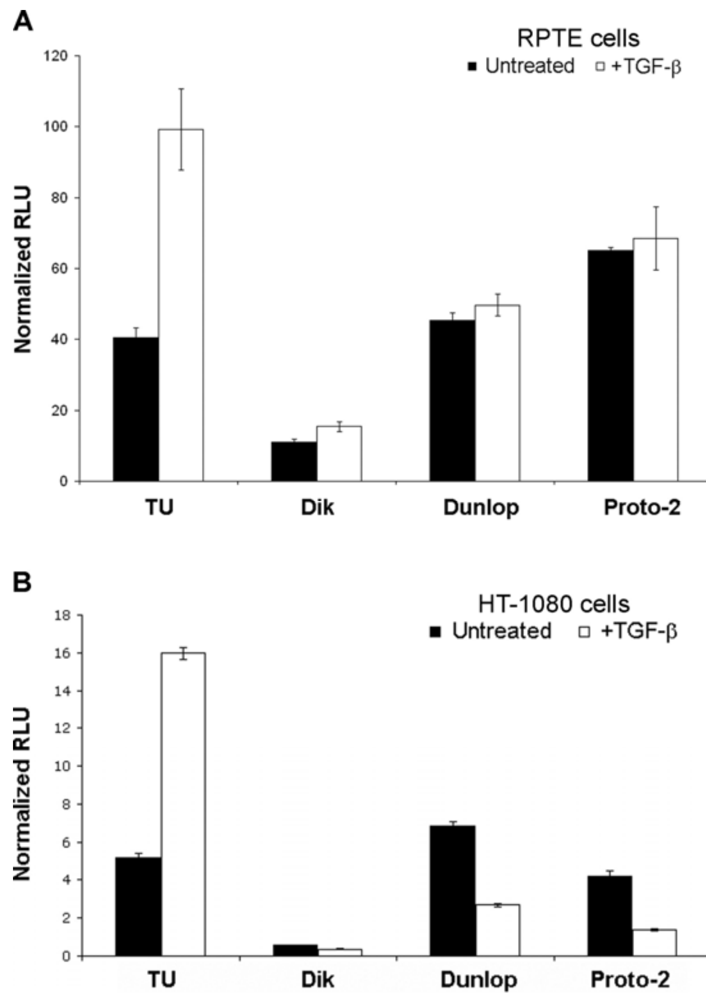

Figure 3.2. BKV early promoter activity in the presence of TGF-β. A) RPTE cells were cotransfected with BKV early promoter-firefly luciferase constructs and a promoterless control *Renilla* luciferase plasmid. TGF-β was added at three to four hpt and total cell lysates were harvested at 48 hpt. Luciferase assays were performed on triplicate samples and data are represented as relative light units (RLU) of firefly luciferase activity, normalized to RLU of *Renilla* luciferase activity. Data shown represent results obtained from three independent experiments. B) HT-1080 cells were cotransfected with BKV early promoter-firefly luciferase constructs and a promoterless control *Renilla* luciferase plasmid and assayed as described in (A). Data shown represent results obtained from three independent experiments.

activity was upregulated by 3.1-fold ($P < 0.001$) and Dik activity remained relatively unchanged (1.6-fold decrease, $P < 0.001$) in the HT-1080 cells. However, the Dunlop and Proto-2 promoters were repressed in the presence of TGF-β in HT-1080 cells by 2.6- and 3.1-fold respectively ($P < 0.001$ and $P = 0.002$, respectively). In addition to cells of the kidney and urinary tract, BKV sequences have been isolated from peripheral blood mononuclear cells (Chatterjee et al., 2000; Doerries et al., 1994), tonsils (Goudsmit et al., 1982), and brain (De Mattei et al., 1995; Elsner and Doerries, 1992). Thus, TGF-β-mediated signaling may differentially regulate BKV during infections of other cell types.

Having mapped the effect of TGF-β to the BKV promoter, we wanted to examine the differences between the TU NCCR and the other three NCCRs used in our studies. We performed an alignment of the four BKV NCCRs and observed that there was one region (7 bp, starred nucleotides) of the TU NCCR that did not align to any region of the Dik, Dunlop, or Proto-2 NCCRs (Figure 3.3). We used the MatInspector transcription factor binding site prediction program (Cartharius et al., 2005) to analyze the potential binding sites in the viral promoters. Within this unique sequence in the TU NCCR, the program predicted a binding site for the transcription factor ZEB-1/AREB6 with a high probability. There were no predicted ZEB-1 binding sites in the other three NCCRs. ZEB-1 has been reported to interact with Smad3 and mediate TGF-β-dependent gene regulation (Postigo, 2003; Postigo et al., 2003). Interestingly, in both the TU and Dik NCCRs the MatInspector program also predicted an adjacent binding site for the transcription factor Smad3 with a high probability. The proximity of the predicted

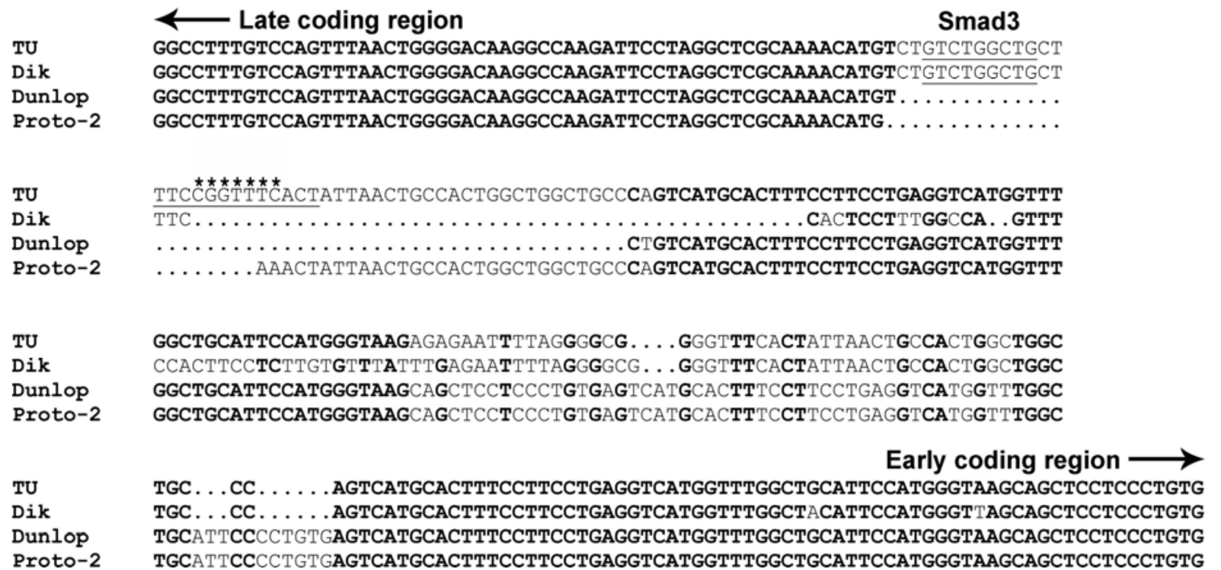

Figure 3.3. Alignment of BKV NCCRs. The segments of the NCCRs (TU, Dik, Dunlop, Proto-2) from the nucleotide before the start codon of agnoprotein through the P block directly adjacent to the O block are shown. The O block is not shown because it is highly homologous between strains. Bold type indicates nucleotides that are identical in at least three of the strains. Underlined regions indicate the predicted Smad3 and ZEB-1 binding sites. Dots indicate nucleotides not found within a sequence. The starred nucleotides indicate the region of the TU NCCR that does not align anywhere in the Dik, Dunlop, or Proto-2 NCCRs. The early promoter is read from left to right (top to bottom), in the direction of the early coding region. The late promoter is read from right to left (bottom to top), in the direction of the late coding region.

ZEB-1 site to the predicted Smad3 site in the TU NCCR suggests that a Smad3-ZEB-1 complex could actively bind to the promoter and regulate early gene transcription.

TU promoter sequences required for TGF-β-mediated regulation. We used site-directed mutagenesis to modify the luciferase plasmids to define the exact sequences required for TGF-β-dependent activation of the early promoter (Figure 3.4A). Starting with the pGL2-TU plasmid as a template, we introduced a single base change (C to G) in the core of the predicted Smad3 binding site, resulting in the pGL2-TU-Smt plasmid. This mutation has previously been shown to effectively disrupt the ability of Smad proteins to bind to the DNA (Jonk et al., 1998). In a similar manner, we modified the pGL2-Dik plasmid, which contains a predicted Smad3 binding site identical to that found in the TU NCCR, lacks a predicted ZEB-1 binding site, and is not affected by TGF-β. Using site-directed mutagenesis, we introduced six nucleotides (GGTTTC) to place a predicted ZEB-1 binding site in the same relative position and orientation as in the TU NCCR (pGL2-Dik+6TU).

The wildtype and mutated luciferase reporter plasmids were transfected into RPTE cells in the absence or presence of TGF-β, and lysates were assayed for luciferase activity at 48 hpt (Figure 3.4B). Similar to previous results, the TU early promoter was upregulated by 2.2-fold ($P = 0.009$) in the presence of TGF-β, while the Dik promoter showed little change in activity (1.2-fold increase). The mutant TU promoter was also unresponsive to TGF-β treatment (1.2-fold decrease), suggesting that the putative Smad3 site is required for TGF-β-dependent promoter activation and that the putative ZEB-1 site alone is not sufficient. In addition, the mutant Dik promoter was upregulated by 2.7-fold ($P = 0.002$) in the presence of TGF-β, suggesting that both the predicted Smad3 and

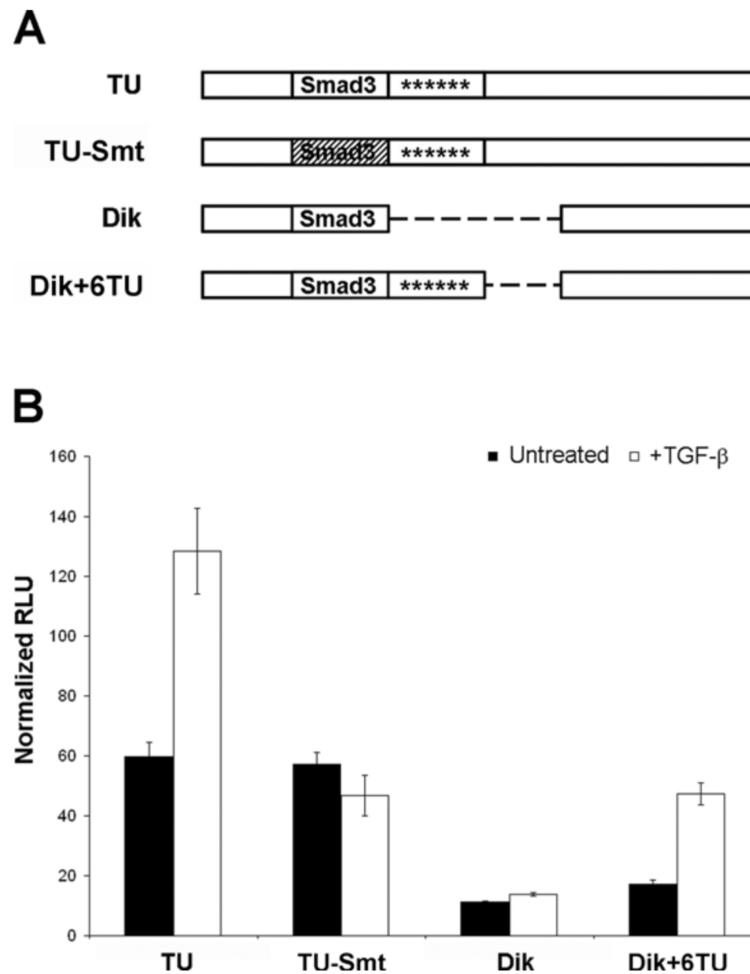

Figure 3.4. Regions of the TU promoter required for TGF-β-mediated regulation of BKV. A) Schematic representation of promoter constructs. TU-Smt has a single base change in the core of the predicted Smad3 binding site (hatched). Dik+6TU has a 6 bp insertion that creates the predicted ZEB-1 binding site. The early promoter is read from left to right, with the start codon for the firefly luciferase gene following the right end of the promoter. B) RPTE cells were cotransfected with wildtype or mutant BKV early promoter-firefly luciferase constructs and a promoterless control *Renilla* luciferase plasmid. TGF-β was added at three to four hpt and total cell lysates were harvested at 48 hpt. Luciferase assays were performed on triplicate samples and data are represented as relative light units (RLU) of firefly luciferase activity, normalized to RLU of *Renilla* luciferase activity. Data shown represent results obtained from three independent experiments.

ZEB-1 sites are necessary for upregulation. It is important to note that the mutations did not significantly change the basal activities of the promoters (Figure 3.4B: compare untreated, TU to TU-Smt; untreated, Dik to Dik+6TU). These results suggest that regulation depends directly on the presence of binding sites for transcription factors involved in TGF-β signaling in the viral early promoter, and that the absence of such sites results in unresponsiveness to TGF-β.

The MatInspector binding site predictions and the results of the luciferase assays strongly suggested that Smad3 and ZEB-1 regulate the TU promoter. However, our attempts to show Smad3 and ZEB-1 binding to the viral promoter using three different assays, electrophoretic mobility shift assay (EMSA), anchored transcriptional promoter assay (Ravichandran and Major, 2006), and siRNA-mediated knockdown of Smad3 and ZEB-1, failed to demonstrate conclusively Smad3 and ZEB-1 binding to, or activation of, the TU promoter in the presence of TGF-β (data not shown). It remains possible that Smad3 is acting with another transcription factor, or that the assays simply were not well suited for demonstration of binding of these particular factors in our system. It will be of interest to define the nature of the factors mediating the TGF-β response in the future.

Previous studies have shown the relevance of Smad-dependent TGF-β signaling during viral infections. For example, JC virus (JCV)-infected oligodendrocytes have elevated levels of TGF-β, Smad3, and Smad4, and chloramphenicol acetyl transferase assays demonstrate the activating effect of Smad protein overexpression on the early and late viral promoters (Enam et al., 2004). Recently, it was shown that TGF-β stimulates JCV replication and that MAPK kinase (MEK) inhibitors can block this effect, indicating a role for activated downstream transcription factors in TGF-β-mediated upregulation

(Ravichandran et al., 2007). Epstein-Barr virus (EBV), a ubiquitous human gammaherpesvirus that, like BKV, has both latent and lytic stages of infection, is also regulated by TGF-β-mediated signaling. Studies have shown that TGF-β treatment drives the reactivation of EBV lytic infection from latently-infected B cells (di Renzo et al., 1994) and epithelial cells (Fukuda et al., 2001). These and other reports have established the relevance of TGF-β and Smad3 in viral regulation (Li et al., 2006; Reed, 1999).

Our studies of cytokine-mediated regulation of BKV gene expression and replication are targeted at understanding the process of viral reactivation in immunosuppressed transplant patients. Previously, it has been shown that common immunosuppressive therapies used in renal transplantation patients, such as cyclosporine and tacrolimus, result in upregulation of TGF-β expression in various cell types, including kidney epithelial cells (Khanna et al., 1999a; Khanna et al., 1999b; McMorrow et al., 2005; Shihab et al., 1996). Therefore, we hypothesized that TGF-β-mediated upregulation of the TU strain of BKV would result in an enhanced ability to reactivate in renal transplant recipients and, consequently, an increase in virulence of this particular strain. However, there are few reports of the TU strain in clinical isolates (Sundsfjord et al., 1994; Sundsfjord et al., 1990), and we have been unable to identify other naturally occurring strains of BKV that contain both the sequences in the TU promoter that are required for TGF-β-mediated activation. Nevertheless, the predicted Smad3 site occurs in a number of other strains [MAN10B (GenBank accession no. **DQ176633**), WWT (**M34048**), URO1 (**U33549**), TC-3 (**AF164514**), AS (**M23122**), TW-2 (**AB213487**), SJH85B (**DQ176634**), T2R.1BKreg-3-4 (**AF442893**), SA090600 (**AF356532**)], and

Smad3 is known to have many different functional binding partners, including AP-1, c-MYC, NF-κB, and Sp1 (Brown et al., 2007), all of which also have binding sites in the NCCR. The results of the luciferase assays using mutated promoters (Figure 3.4) support the likelihood that there are two transcription factors involved in regulating the response to TGF-β. Thus, there is potential for TGF-β-mediated activation or repression of other BKV strains.

Our studies provide evidence for the transcriptional regulation of BKV early gene expression by as yet unidentified components of the TGF-β signaling cascade. However, the postulated contribution of TGF-β to BKV reactivation in transplant patients must be considered in the context of the overall immune status of those individuals. Previously, we also described the inhibition of BKV replication by IFN-γ, in a strain-independent manner (Abend et al., 2007). It is therefore reasonable to posit that reactivation of BKV in immunocompromised patients may require multiple signals, such as the enhancement of viral gene expression by TGF-β and the absence of IFN-γ-mediated inhibition of replication, as well as others. Understanding the contributions of each signal to the outcome of the infection will be critical for building a complete picture of the interaction between BKV and the host immune system.

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CHAPTER IV

PRELIMINARY RESULTS ON THE CHARACTERIZATION OF INTERFERON-GAMMA-MEDIATED REGULATION AND ARCHETYPE BK VIRUS REPLICATION IN A TISSUE CULTURE SYSTEM

BK virus (BKV) is a ubiquitous pathogen that infects nearly the entire population by the age of 10 (Knowles, 2001; Knowles et al., 2003). The transmission of BKV is not yet characterized but is thought to occur by either the respiratory or urino-oral route. Following a typically subclinical primary infection, BKV is able to disseminate and establish an infection of kidney epithelial cells, particularly proximal tubule epithelial cells, and the urothelium (Chesters et al., 1983; Heritage et al., 1981). It is within these cells that the virus persists throughout the life of the host. Although BKV does not typically cause disease in healthy individuals, the virus is periodically shed in the urine (Knowles, 2001; Zhong et al., 2007). In immunosuppressed patients, BKV can cause severe disease by reactivating to a robust lytic infection. Approximately 10% of renal transplant recipients undergo BKV reactivation that develops into polyomavirus nephropathy (PVN), a lytic infection of kidney epithelial cells that can result in loss of function or destruction of the graft. In addition, approximately 10% of bone marrow transplant recipients suffer from BKV reactivation, resulting in hemorrhagic cystitis (HC), a painful infection of bladder epithelial cells characterized by hematuria.

BKV is a member of the polyomavirus family and is highly homologous to JC virus (JCV), the other human polyomavirus, and SV40, the well-studied simian virus (Cubitt, 2006; Imperiale, 2001). BKV has a small (40-45 nm), nonenveloped icosahedral virion and a circular, double-stranded DNA genome of approximately 5.2 kb. The genome can be divided into three major regions: the early coding region, which contains the genes for large tumor antigen (TAg), small tumor antigen (tAg), and truncated tumor antigen (truncTAg; D. Das, A. Joseph, J. Abend, D. Campbell-Cecen, and M. Imperiale, in preparation); the late coding region, which contains the genes for capsid proteins VP1, VP2, and VP3, and agnoprotein, which does not yet have a defined function; and the non-coding control region (NCCR), which contains the origin of replication and the early and late promoters, which function in a bidirectional manner. In the nucleus of the infected cell, BKV genomic DNA becomes associated with the cellular histones H2A, H2B, H3 and H4 to form a viral minichromosome that is then packaged into virions (Meneguzzi et al., 1978).

This chapter will describe preliminary results from two areas of research that follow from the work presented in Chapters II and III. The first area of research is a continuation of the study of interferon-gamma (IFN-γ) mediated inhibition of BKV replication, with a focus on the characterization of chromatin remodeling events. The second area of research is aimed at identifying factors that are required for archetype BKV strains to replicate in a tissue culture system. It is possible that our findings in this area will also be linked to chromatin remodeling events, implicating chromatin structure as an important mechanism for regulating BKV replication.

Materials and Methods

Cell culture and reagents. Primary human renal proximal tubule epithelial cells (RPTE cells, Cambrex) were maintained for up to six passages in renal epithelial cell basal medium (REBM, Cambrex) supplemented with human epidermal growth factor, fetal bovine serum (FBS), hydrocortisone, epinephrine, insulin, triiodothyronine, transferrin, and GA-1000 as indicated for renal epithelial cell growth medium (REGM, supplements obtained as REGM SingleQuots, Cambrex). RPTE cells were grown at 37°C with 5% CO_2 in a humidified incubator. Recombinant human IFN-γ (Peprotech, Inc) was reconstituted according to manufacturer's recommendations and used at 50 U/ml or 250 U/ml. Trichostatin A (TSA, Sigma) and sodium butyrate (NaB, Sigma) were reconstituted according to manufacturer's instructions and used at 1.32 μM and 5 mM, respectively.

Viruses and infections. The genome of BKV strain TU was cloned into pGEM-7zf(-) at the EcoRI site. The genome of BKV strain Dik (archetype) was cloned into pBR322 at the BamHI site (gift of J. Lednicky). BKV TU viral stocks were prepared and titrated as previously described (Abend et al., 2007). RPTE cells were infected at 70% confluence with BKV TU at an MOI of 0.5 IU/cell (infectious units per cell) and incubated at 37°C for one hour. The viral lysate used for infection was then replaced with fresh REGM. The dose and time of treatment with IFN-γ, TSA, or NaB is described in the figure legends.

Transfections. BKV genomic clones were prepared for transfection as follows: pGEM-7-TU and pBR322-Dik were digested with EcoRI or BamHI, respectively, recircularized

with T4 DNA ligase, and phenol-chloroform extracted. RPTE cells were transfected at 50% confluence using Effectene (Qiagen) and the following conditions: 0.6 μg DNA per well of a 12-well plate with a DNA to Effectene ratio of 1:20. In the cotransfection experiment (Figure 4.7), cells were transfected with 0.5 μg of BKV genomic DNA and 0.1 μg of the TAg expression plasmid (pcDNA3.1 with TAg cDNA cloned into the BamHI and EcoRV sites).

Low molecular weight DNA preparations. Low molecular weight DNA was isolated from cells using a modified Hirt protocol (Hirt, 1967). Cells were collected and pelleted at 300 x g for 5 min at 4°C. Cell pellets were resuspended in Lysis buffer (0.6% SDS, 10 mM EDTA) and incubated at room temperature (RT) for 20 min. Sodium chloride (NaCl) was added to a final concentration of 1.4 M and the samples were incubated overnight at 4°C to precipitate the cellular genomic DNA, which was then pelleted at 20,000 x g for 20 min at 4°C. The supernatants were incubated with 1.5 μg/μl Pronase E and 150 ng/μl RNAse A for one hour at 37°C. Samples were phenol-chloroform extracted three times and precipitated overnight at -80°C with 95% ice cold ethanol and 0.3 M sodium acetate. The low molecular weight DNA was pelleted at 20,000 x g for 10 min at 4°C, washed in 70% RT ethanol, and resuspended in 15 μl of nuclease-free water. Nuclear low molecular weight DNA was prepared by first isolating nuclei from infected cells using the Dignam protocol (Dignam et al., 1983). Pelleted nuclei were treated as a cell pellet and the modified Hirt protocol was performed as described above.

Real time quantitation of viral genomes. Low molecular weight DNA samples prepared for this analysis were spiked with 500 ng pRL-Null plasmid (Promega) directly after incubation in Lysis buffer to control for sample loss during the procedure. Primers

were designed using Primer3 software (Rozen and Skaletsky, 2000) to amplify 125- and 129-bp fragments of the BKV TU NCCR and β-lactamase gene of pRL-Null (Promega), respectively. Primers were synthesized by Invitrogen and the sequences are as follows: TUNCCRFOR (5' CGCCCCTAAAATTCTCTCTT 3'); TUNCCRREV (5' ATGTCTG TCTGGCTGCTTTC 3'); RTAmpFOR (5' TCGCCGCATACACTATTCTC 3'); RTAmpREV (5' GCCGCAGTGTTATCACTCAT 3'). Reactions were performed in a total volume of 25 μl using *Power* SYBR Green PCR Master Mix (Applied Biosystems), 1 μl 1:125 diluted sample, and 300 nM of each primer. Amplification was performed in 96-well PCR plates (Bio-Rad) using the iCycler iQ5 Real Time Detection System (Bio-Rad) with the following PCR program: 2 min at 50°C; 10 min at 95°C; 40 cycles of denaturation at 95°C for 15 sec and annealing and extension at 58°C for 1 min. Results are presented as the fold change in BKV genome copy numbers, with the levels in the "36 hpi, IFN-γ, Nuc" sample arbitrarily set to one. Results were normalized to levels of pRL-null (internal plasmid control) using the Livak method (Livak and Schmittgen, 2001).

Western blotting. Total cell protein was harvested at the indicated times post-infection using EIA lysis buffer (Harlow et al., 1986) supplemented with 5 μg/ml phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 0.05 M sodium fluoride, and 0.2 mM sodium orthovanadate. Samples were electrophoresed, transferred to a nitrocellulose membrane, and probed with antibodies as previously described (Abend et al., 2007). The following primary antibodies were used: pAb416 (Harlow et al., 1981) for detection of TAg expression, p5G6 (gift of D. Galloway) for detection of VP1 expression, #9171 (Cell Signaling Technology) for detection of phospho-STAT1 levels,

and Ab8245 (Abcam) for detection of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression.

Southern blotting. Low molecular weight DNA samples were prepared for electrophoresis by digestion with EcoRI to linearize the viral genome and DpnI, which only cuts methylated sequences, to distinguish input DNA from the replicated genome. Samples were electrophoresed on a 1% agarose gel at 100 V and transferred to a positively charged nylon membrane (Perkin Elmer) by capillary action overnight in 20x SSC (3 M NaCl, 0.3 M sodium citrate dihydrate). The probe was prepared by digestion of pGEM-7-TU with PvuII and isolation of the 3.2 kb fragment corresponding to the early coding region. The Random Primers DNA Labeling System (Invitrogen) was used to radiolabel 100 ng of the PvuII fragment with [α-^{32}P]dCTP and 20 ng of probe was added to the hybridization buffer (5x SSC, 1% SDS, 1x Denhardt's solution, 100 µg/ml denatured salmon sperm DNA) for incubation overnight at 68°C. The blot was then washed extensively and exposed to film.

Site-directed mutagenesis and NCCR swap. The following primers were synthesized and HPLC purified (Invitrogen) to introduce bases changes (in bold) that insert restriction enzyme sites (underlined) into the BKV genomic clones: NCCRSpeIFOR (5' GGGGA AATC**A**CTA**G**TCTTTTGCAATTTTTGCAAAAATGG 3'); TAgPmlIFOR (5' ACACCACCCCCAAAATAACAC**GT**GCTTAAAAGTGGCTTATAC 3'); NCCRSacIIFOR (5' GACAAGGCCAAGATTCC**GC**GGCTCGCAAAACATGTC 3'). The reverse primers are exactly the reverse complement of the sequences shown. Mutagenesis was performed according to the protocol for the Quik Change II Site-Directed Mutagenesis Kit (Stratagene) using primer pairs at 1.25 nM each, 100 ng of

pGEM-7-TU or pBR322-Dik as template, 1 mM dNTPs, and 1.25 U Native Pfu DNA polymerase (Stratagene) in 25 μl total reaction volume. The following two-step PCR program was used: 3 min at 95°C, 18 cycles of denaturation at 95°C for 15 sec and annealing and extension at 65.5°C for 16 min and 30 sec. The resulting genomic clones were digested with SpeI and SacII and both the plasmid and NCCR fragments were isolated by gel extraction. NCCRs were religated into the opposite genomes and the DNA was prepared for transfection as described above.

Preliminary Results on the Characterization of IFN-γ-Mediated Regulation

IFN-γ is a widely known anti-viral cytokine produced primarily by T cells and NK cells. It is a hallmark of the T helper 1 (Th1) phenotype of T cells and overall has pro-inflammatory, anti-proliferative, and pro-apoptotic effects on cells. The IFN-γ receptor is expressed on most cell types, including the kidney epithelial cells used in our studies. The signaling cascade is initiated by binding of IFN-γ to its cell surface receptor, which results in the phosphorylation of Janus kinase 1 (JAK1) and JAK2, which are associated with the cytoplasmic domains of the receptor. Activated JAK1 and JAK2 then phosphorylate the receptor, which allows recruitment and binding of signal transducer and activator of transcription 1 (STAT1). Upon binding the receptor, STAT1 is phosphorylated, which promotes dimerization and translocation to the nucleus. Activated STAT1 homodimers act as transcription factors in the nucleus to mediate expression of IFN-γ responsive genes (reviewed in Pestka et al., 2004; van Boxel-Dezaire and Stark, 2007).

In microarray studies, IFN-γ stimulation has been shown to significantly induce the expression of over 100 genes at early times post-treatment, including a number of transcription factors such as STAT1, IRF-1, PML, IRF-9, C/EBP, and TEAD4 (Der et al., 1998). In addition, IFN-γ signaling promotes the activation of many other factors, including STAT3, AP-1, USF-1, NF-κB, IRF-1, IRF-8, ATF-2, GATA-1, CREB, and PU.1 (van Boxel-Dezaire and Stark, 2007). These transcription factors can subsequently regulate the expression of other proteins in a second wave of IFN-γ-mediated transcription (van Boxel-Dezaire and Stark, 2007).

Transcription factors are DNA binding proteins that regulate transcription mainly by binding to enhancer regions, recruiting other cellular factors and facilitating the formation of a stable transcription initiation complex at the promoter of a gene. Transcription factors can also regulate gene expression by binding and recruiting chromatin remodeling enzymes, histone acetyltransferases (HATs) and histone deacetylases (HDACs, reviewed in Bhaumik et al., 2007; Struhl, 2006). The N-terminal tails of histones contain several lysine residues, which have positive charges that interact with the negatively charged backbone of DNA, forming a closed, condensed chromatin structure. HATs transfer acetyl groups (-$COCH_3$) from acetyl-coenzyme A to histone lysine residues, resulting in the neutralization of charged histone tails and an opening of the chromatin structure. There are many known HATs, including p300/CBP, P/CAF, Gcn5, ACTR, SRC-1, and TAF130/250. Hyperacetylated histones are typically associated with genes that are being actively transcribed. HDACs are enzymes that remove acetyl groups from the lysine residues, resulting in a stronger interaction between histones and DNA and subsequently condensing the chromatin. There are four classes of HDACs, with class I (HDAC1, -2, -3, and -8) being the most widely expressed (Adcock, 2006). Hypoacetylated histones are usually associated with genes that have been silenced. Histones can also be ubiquitinated, phosphorylated, methylated, and sumoylated; all of these modifications can affect chromatin structure by altering the interaction between histones and DNA (Bhaumik et al., 2007).

In Chapter II, we described the inhibitory effect of IFN-γ on BKV replication, mediated primarily at the level of early gene transcription. The inhibition of TAg expression results in repression of viral DNA replication, late gene expression, and

progeny production due to the integral role of TAg in the viral life cycle. We propose that there are several mechanisms by which IFN-γ could affect viral replication. First, it is possible that IFN-γ disrupts viral trafficking during entry or delivery of the viral genome to the nucleus. More likely, we hypothesize that IFN-γ signaling has a direct effect on transcription initiation by either 1) activating transcriptional repressors that bind viral DNA and prevent formation of the transcription initiation complex, 2) activating transcription factors that recruit HDACs and facilitate the condensation of the viral genome, or 3) activating transcriptional repressors that prevent expression or activation of HATs. In this chapter, we will first present data demonstrating that IFN-γ does not affect delivery of the viral genome to the nucleus, suggesting instead a direct effect on transcription initiation. In addition, we will show that the effect of IFN-γ is long-lasting and reversible by simultaneous treatment with HDAC inhibitors. While more studies are needed, these results suggest that IFN-γ mediates chromatin remodeling events on the BKV minichromosome.

Results and Discussion

As stated above, an alternative explanation for the observed effects of IFN-γ on BKV replication (see Chapter II) is that IFN-γ signaling inhibits viral trafficking or delivery of genomic DNA to the nucleus, the site of viral replication. To rule out this possibility, RPTE cells were infected with BKV and treated with IFN-γ at 3 hours post-infection (hpi). Low molecular weight DNA was harvested either from whole cells or from isolated nuclei at 36 hpi, approximately when TAg expression is first detectable but prior to viral DNA replication, and at 96 hpi, a late stage of infection when viral progeny

are being produced and released from cells. Samples were analyzed in a real time PCR assay to quantify the viral genome copies present (Figure 4.1). At 36 hpi, there was no difference in viral genome copy number in the nuclei of untreated and IFN-γ treated cells, indicating that IFN-γ has no effect on viral trafficking or DNA delivery to the nucleus. As expected, there were more viral genomes present in the whole cell sample than in the nuclear sample (compare 36 hpi UN, Total and 36 hpi UN, Nuc), indicating that a fraction of viruses had entered the cell but not yet trafficked to the nucleus. At 96 hpi, there was a 9.8-fold decrease in the number of viral genomes present in nuclei of IFN-γ treated cells compared to untreated cells. This result is comparable to our previous observations of IFN-γ inhibition of TAg transcript levels and protein expression (Chapter II). Thus, IFN-γ does not appear to affect viral trafficking, but instead mediates an inhibitory effect on BKV early region transcription, consequently leading to a decrease in gene expression and viral DNA replication.

Previously, we observed an interesting pattern of viral gene expression in the presence of IFN-γ: TAg and VP1 levels peaked at 72 and 84 hpi, respectively, after which expression tapered down to a low level. To examine the extent of this effect, RPTE cells were infected, treated with IFN-γ at 3 hpi, and total cell lysates were harvested at 5, 7, 9, 11, and 13 days post-infection (dpi). Western blot analysis revealed that this low level of viral gene expression was maintained throughout the duration of the experiment, even though the cells were only treated once with IFN-γ (Figure 4.2). We would not expect a cytokine to be stable for this length of time in the media, however it is possible that the signaling cascade is being activated by the crosstalk between the type I and type II interferon pathways (Pestka et al., 2004; van Boxel-Dezaire and Stark, 2007).

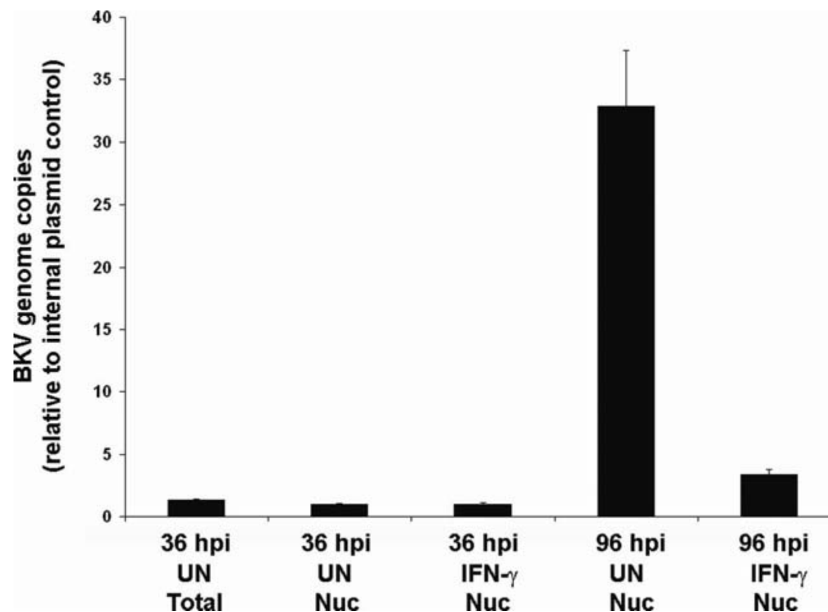

Figure 4.1. IFN-γ does not affect delivery of BKV DNA to the nucleus. RPTE cells were infected with BKV TU at an MOI of 0.5 IU/cell and treated with 250 U/ml IFN-γ at three hpi. Low molecular weight DNA was harvested at 36 or 96 hpi from whole cells (Total) or isolated nuclei (Nuc). Samples were analyzed by real time PCR to determine the relative number of BKV genomes in each sample. Results are presented as fold change in BKV genome copy numbers, normalized to the levels of an internal control plasmid to account for sample loss during preparation. Samples were analyzed in triplicate and the fold change in BKV genome copies at 36 hpi in the nuclei of cells treated with IFN-γ (36 hpi, IFN-γ, Nuc) was arbitrarily set to one. UN, untreated; hpi, hours post-infection.

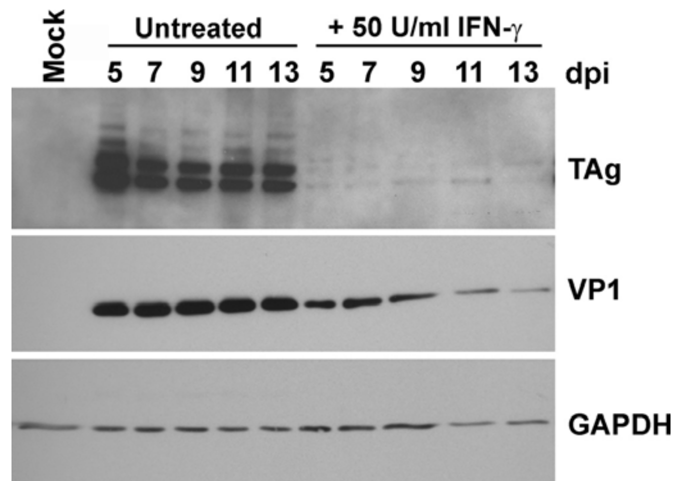

Figure 4.2. IFN-γ-mediated inhibition of viral gene expression is sustained out to late stages of infection. RPTE cells were infected with BKV TU at an MOI of 0.5 IU/cell and treated with 50 U/ml IFN-γ at three hpi. Total cell lysates were harvested at 5, 7, 9, 11, and 13 dpi. For each sample, 5 μg of protein were electrophoresed on an 8% SDS-polyacrylamide gel and analyzed by Western blot, probing for TAg, VP1, and GAPDH. Mock, mock-infected samples with no IFN-γ treatment; dpi, days post-infection.

Alternatively, this result may indicate that histones associated with the viral minichromosome are being deacetylated, which would reduce viral gene expression and may require a positive signal for initiation of HAT activity. In this scenario, the viral genome would appear to be stably repressed in the presence of IFN-γ.

We next postulated that pretreatment of cells with IFN-γ would induce an anti-viral state such that viral gene expression would be inhibited from the time of genome delivery to the nucleus. In this experiment, cells were either untreated, treated with IFN-γ at 3 hpi, treated at 3 hpi and washed at 24 hpi to remove IFN-γ, pretreated for 24 h with no treatment after infection, or pretreated for 24 h with IFN-γ added after infection and washed away at 24 hpi. Total cell lysates were harvested at 5 dpi and analyzed by Western blot probing for TAg, VP1, phosphorylated STAT1, and GAPDH (Figure 4.3). It appears that any exposure to IFN-γ is sufficient for repression of TAg expression. There is a clear difference, however, in the levels of VP1 such that pretreatment appears inhibit expression more than treatment at 3 hpi. Phosphorylated STAT1 levels were examined to monitor the activity of the IFN-γ signaling cascade. Interestingly, the samples in which washes were performed to remove IFN-γ from the cells were very similar to lysates from unwashed cells, indicating a continued activation of this signaling cascade at 5 days post-treatment. The persistence of the IFN-γ signaling cascade could explain the prolonged effect we see on viral gene expression.

To determine if IFN-γ regulates BKV through HDAC activity, cells were infected and then treated simultaneously at 1 hpi with IFN-γ and either trichostatin A (TSA) or sodium butyrate (NaB), two broad-spectrum HDAC inhibitors. Total cell protein or low molecular weight DNA was harvested at 4 dpi and analyzed by Western or Southern blot,

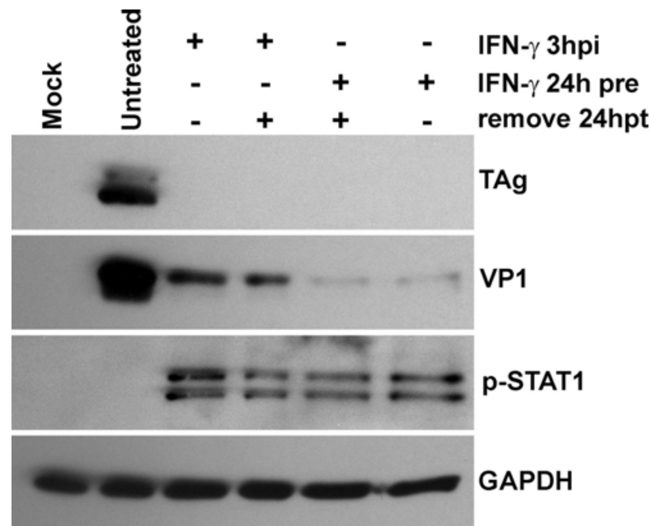

Figure 4.3. Pretreatment with IFN-γ results in greater inhibition of gene expression. RPTE cells were infected with BKV TU at an MOI of 0.5 IU/cell and treated with 250 U/ml IFN-γ in the following ways: treated at 3 hpi, treated at 3 hpi and washed at 24 h post-treatment, pretreated for 24 h, or pretreated for 24 h, treated again directly after infection, and washed at 24 h post-treatment. Total cell lysates were harvested at 5 dpi and 8 μg of protein were electrophoresed on an 8% SDS-polyacrylamide gel and analyzed by Western blot, probing for TAg, VP1, phosphorylated STAT1, and GAPDH. Mock, mock-infected samples with no IFN-γ treatment; hpi, hours post-infection; dpi, days post-infection; IFN-γ 24h pre, pretreated cells for 24 h with IFN-γ; remove 24hpt, washed cells at 24 h post-treatment.

respectively (Figure 4.4). TSA treatment alone resulted in increased TAg expression; however, in cells treated with both TSA and IFN-γ, the levels of TAg were similar to those of untreated cells. The same result was seen in the Southern blot analysis for viral DNA replication, indicating that TSA treatment was able to prevent IFN-γ-mediated inhibition of BKV. These data suggest that histones associated with the viral genome are being deacetylated in the presence of IFN-γ. In contrast, NaB had little effect on TAg expression but seemed to strongly inhibit viral DNA replication both in the absence and presence of IFN-γ. TSA is a hydroxamic acid compound with potent activity against class I and class II HDACs, while NaB is a short chain fatty acid with a lower potency and non-specific inhibitory action against HDACs (Adcock, 2006). Assuming that TSA is acting more specifically on HDACs, these data suggest that IFN-γ inhibition of BKV is HDAC-dependent. Alternatively, NaB appears to mediate a decrease in the levels of modified forms of TAg (various higher molecular weight bands seen in untreated and TSA-treated lanes, Figure 4.4). Similar modifications have been shown to regulate SV40 TAg functions, and could explain the lack of DNA replication.

So far, these experiments are only suggestive of chromatin remodeling and do not provide information about specific histone modifications or the location of the modified histones on the viral genome. Future studies using electrophoretic mobility shift assays (EMSAs) and chromatin immunoprecipitation (ChIP) will reveal these details. We will not pursue the HDAC inhibitor studies any further for several reasons. First, HDAC inhibitors are known to induce cell cycle arrest and apoptosis (Johnstone, 2002; Richon et al., 2000), and thus non-specific effects may confound results after prolonged exposure. Second, there are reports that HDACs are required for IFN-γ-mediated

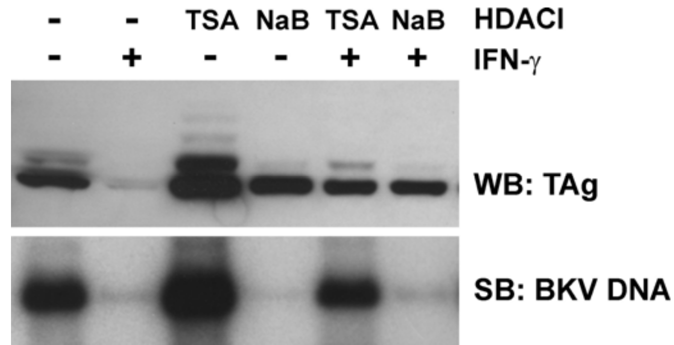

Figure 4.4. Treatment with HDAC inhibitors restores BKV gene expression and replication in the presence of IFN-γ. RPTE cells were infected with BKV TU at an MOI of 0.5 IU/cell and treated with 250 U/ml IFN-γ and/or 1.32 μM trichostatin A (TSA) or 5 mM sodium butyrate (NaB) at 1 hpi. Total cell lysates or low molecular weight DNA were harvested at 4 dpi. To assay for early gene expression, 8 μg of protein were electrophoresed on an 8% SDS-polyacrylamide gel and analyzed by Western blot, probing for TAg. To assay for viral DNA replication, low molecular weight DNA samples were run on a 1% agarose gel and analyzed by Southern blotting as described in Materials and Methods. HDACI, histone deacetylase inhibitor; WB, Western blot; SB, Southern blot.

signaling events, and that HDAC inhibitors are known to interfere with IFN-γ signaling by preventing STAT1 phosphorylation, nuclear translocation and gene regulation (Klampfer et al., 2004; Nusinzon and Horvath, 2005). Although treatment with HDAC inhibitors has not affected phosphorylation of STAT1 in our studies (data not shown), we only intended to use this approach as a preliminary screen for HDAC activity in the presence of IFN-γ.

The large number of genes activated and regulated by IFN-γ signaling makes it difficult to hypothesize what factors are involved in the inhibition of BKV transcription. While we postulate that the histone modifications will be located within the NCCR, this 400 to 500 bp region contains many predicted and proven binding sites for transcription factors that could recruit HDACs or other modifying enzymes. Our immediate plans involve performing EMSAs with short overlapping probes of the NCCR to map the regions of DNA that bind additional proteins when incubated with nuclear extracts from IFN-γ treated cells. Then, targeted sequence analysis and supershift assays with specific antibodies should allow us to identify the factors involved in the regulation. Concurrently, we will determine the type and location of histone modifications in the presence of IFN-γ using ChIP. Immunoprecipitation with antibodies for specific modified histone residues will indicate the type of modification, while PCR for specific regions of the genome will map the location of the modified histones. These approaches will also allow us to examine other modifications of viral DNA-associated histones, such as methylation, phosphorylation, and ubiquitination.

Chromatin remodeling has been reported as a means to regulate transcription and replication in a number of viral systems. For example, the latent and lytic states of herpes

simplex virus are controlled by remodeling of the viral chromatin (Knipe and Cliffe, 2008). For HIV, histone deacetylation at the LTR is responsible for restriction of Tat expression and transactivation, and thus establishement of latency (Lusic et al., 2003; Treand et al., 2006). The acetylation of histones associated with the SV40 genome has been studied in detail, and appears to play a role in the transition from early to late gene expression (Balakrishnan and Milavetz, 2006; Balakrishnan and Milavetz, 2007). We are interested in the ability of cytokine-mediated signaling to induce chromatin remodeling on the viral genome. We expect that IFN-γ signaling represses BKV gene expression through HDAC activity, making the viral genome less accessible to transcription initiation complexes. In our studies, however, viral gene expression was never completely shut off; there was always a low level of TAg and VP1 expression which could sustain the infection. This could explain the ability of BKV to maintain a persistent infection in the host, with minimal gene expression to avoid detection by the immune response. The reduction in IFN-γ levels by immunosuppression could then allow the viral minichromosome to be opened and transcribed. It is possible that a positive signal is required for the activation and recruitment of HATs to the viral genome, which would fit with the increasingly complex set of risk factors for BKV reactivation in immunosuppressed patients.

Preliminary Results on Archetype BK Virus Replication in a Tissue Culture System

There are two major types of BKV strains, archetype and rearranged, distinguished from each other by NCCR structure. In archetype strains, the NCCR is divided into five blocks of DNA sequences arbitrarily designated O, P, Q, R, and S (Markowitz and Dynan, 1988; Rubinstein et al., 1987; Sundsfjord et al., 1994). The O block contains the origin of replication and the TAg binding sites, while the other blocks contain many transcription factor binding sites and were defined by the apparent ability to move together during NCCR rearrangements. The rearranged strains have NCCR structures that contain partial or full duplications or deletions of these blocks. Rearranged BKV strain NCCRs always have O and S blocks and at least one P block, but the Q and R blocks are frequently deleted and additional P blocks are often present (Cubitt, 2006; Johnsen et al., 1995; Moens and Rekvig, 2001; Moens and Van Ghelue, 2005).

It was previously shown that the archetype BKV strains are functionally different from the rearranged strains. Archetype viruses are more efficient at transforming rodent cells than rearranged viruses, while rearranged strains are far more efficient at replication in tissue culture (Watanabe and Yoshiike, 1982; Watanabe and Yoshiike, 1986). Several groups have tried to propagate archetype BKV in tissue culture by infection of cells with clinical samples or transfection of viral genomic DNA. In each case, the result was either no viral replication or replication with concurrent NCCR rearrangements, indicating contamination of the clinical sample with rearranged virus or, in the case of transfected DNA, the derivation of a rearranged strain from the archetype genome (Rinaldo et al., 2005; Rubinstein et al., 1991; Sundsfjord et al., 1994; Sundsfjord et al., 1990).

Interestingly, the archetype strains are by far the most common BKV strains isolated from clinical samples, both urine and blood of healthy and immunocompromised individuals (Gosert et al., 2008; Markowitz et al., 1991; Negrini et al., 1991; Sharma et al., 2007; Sugimoto et al., 1989; Sundsfjord et al., 1999; Takasaka et al., 2004; ter Schegget et al., 1985). These observations suggest that the archetype strains are not at all defective at replication, but that the appropriate conditions for their propagation have not been attained in tissue culture systems.

Previous studies using *in vitro* reporter assays have demonstrated that the archetype early promoter has lower transcriptional activity than rearranged early promoters (Gosert et al., 2008; Markowitz and Dynan, 1988; Markowitz et al., 1990). Furthermore, BKV promoters with duplications of only the P block have higher transcriptional activity (Chakraborty and Das, 1989; Deyerle and Subramani, 1988). This result could be explained by either the presence of important transcription activator binding sites within the P block, or by the creation of new transcription factor binding sites at the junctions of duplicated blocks. The archetype promoter is not inhibitory to transcription initiation; when short P block fragments from a rearranged NCCR were inserted into an archetype NCCR, the resulting promoter had enhanced transcriptional activity suggesting the absence of inhibitory elements (Markowitz et al., 1990). Although functional for transcription, the lower activity of the archetype early promoter could result in minimal expression of TAg and thus impede the progression of the viral life cycle.

In the second part of this chapter, we begin to explore some of the factors that could be required for archetype BKV propagation. We first propose that the cell types

used thus far are simply not supportive of archetype replication and therefore attempt to productively infect RPTE cells. We also consider the possibility that the archetype genome is somehow more prone to HDAC activity, resulting in the immediate condensation of the viral genome upon entry into a cell and complete repression of viral gene expression. To systematically narrow in on the genomic region that confers the ability of a BKV strain to replicate, we produced chimeric genomes in which the NCCR from an archetype strain was replaced with the NCCR from a rearranged strain. The NCCR swap allowed viral DNA replication of an otherwise archetype genome. Next we examined the functional relevance of the NCCR swap by cotransfecting the archetype genome with a TAg expression plasmid, and again observed viral DNA replication. These results provide information about the conditions in the host that promote archetype BKV strains to replicate.

Results and Discussion

As mentioned above, propagation of archetype BKV has been attempted in several common cell lines, including HUVEC-C, a human umbilical vein endothelial cell line (Rinaldo et al., 2005); Vero, an African green monkey kidney epithelial cell line (Sundsfjord et al., 1990); and HEK, a human embryonic kidney cell line (Rubinstein et al., 1991; Sundsfjord et al., 1990). We first wondered if archetype BKV would replicate in a more relevant cell type, such as the primary RPTE cells characterized by our lab (Low et al., 2004). These cells are major sites of BKV lytic infection in the host during reactivation and PVN, and thus it seemed likely they would readily support archetype replication. RPTE cells were transfected with viral DNA from either a rearranged strain, BKV TU, or an archetype strain, BKV Dik, and viral lysates were harvested at 10 days

post-transfection (dpt). The lysates were then used to infect fresh RPTE cells, which were fixed at 4 dpi and analyzed for TAg expression by immunofluorescence to assay for the presence of infectious viral progeny in the transfection lysates (Figure 4.5). Transfection with the BKV TU genome produced abundant viral progeny, as shown by the numerous TAg positive cells upon infection. Lysates harvested from cells transfected with the archetype genome, however, contained no infectious viral progeny, as indicated by the absence of TAg expressing cells upon infection. The epitope recognized by the monoclonal antibody used in the immunofluorescence assay (WEQ/SWW; Lindner et al., 1998; amino acids 91-95 of TAg), is intact in the TAg sequence of archetype virus, thus these results cannot be explained by an inability of the antibody to recognize infected cells. It is possible, however, that infectious progeny were produced, but that the level of TAg expression upon infection was below the limit of detection of the assay. These results suggest that additional factors are required to drive archetype BKV replication in RPTE cells.

We next wanted to systematically investigate whether different genomic regions affected the ability of BKV strains to replicate. Using site-directed mutagenesis, we inserted unique restriction enzyme sites into archetype and rearranged genomic clones such that the NCCR was flanked by SpeI and SacII sites, and the early coding region was flanked by SpeI and PmlI sites (Figure 4.6A). These three sites will allow us to swap the three major regions of the genome between archetype and rearranged strains.

We began our analysis by swapping the region most likely to affect replication: the NCCR. The resulting chimeric genomes (Arch/TU_N, archetype genome with TU

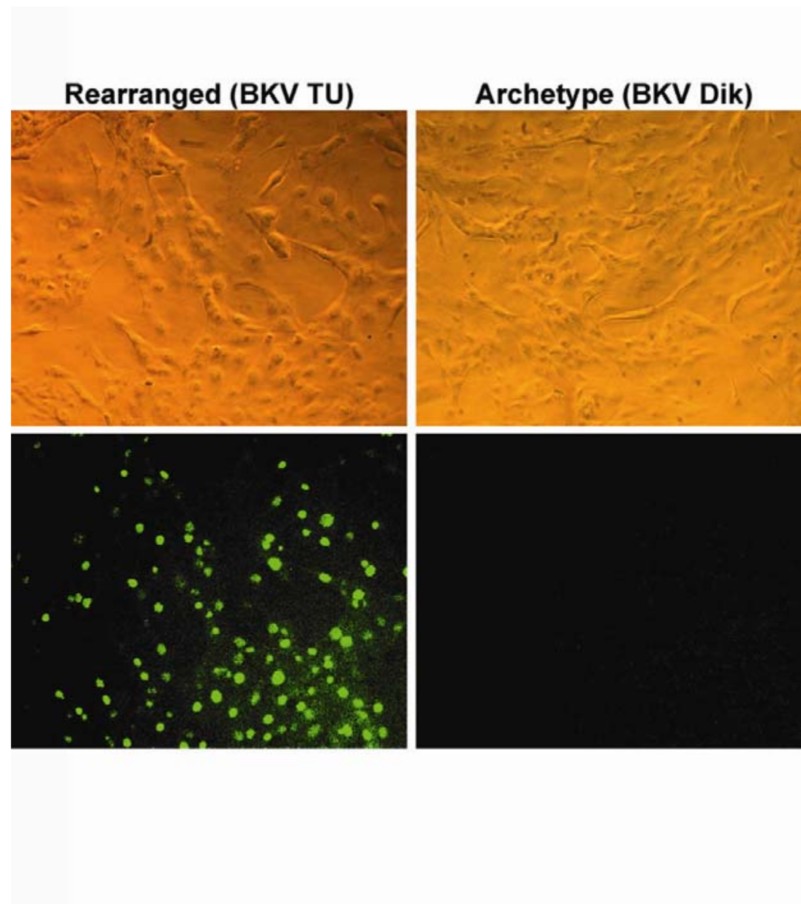

Figure 4.5. Archetype BKV does not productively infect RPTE cells. RPTE cells were transfected with rearranged (BKV strain TU) or archetype (BKV strain Dik) genomic DNA and viral lysates were harvested at 10 dpt. Lysates were subjected to three freeze (-80°C)/thaw (37°C) cycles and used to infect fresh RPTE cells. At 4 dpi, cells were fixed and assayed by immunofluorescence for TAg expression, as described in (Abend et al., 2007). Top panels, brightfield. Bottom panels, FITC channel.

NCCR; TU/Arch$_N$, TU genome with archetype NCCR) were transfected into RPTE cells alongside the original archetype and rearranged genomes. Low molecular weight DNA was harvested at 4 dpt, digested with DpnI, and analyzed by Southern blot to detect replication of viral DNA (Figure 4.6B). In agreement with previous results, we were unable to detect replication of the archetype DNA. The chimeric archetype genome, Arch/TU$_N$, however, replicated robustly in RPTE cells, suggesting that the NCCR is responsible for the block of archetype BKV replication. Similarly, the BKV TU genome was efficient at DNA replication, while the sample harvested from transfection of the chimeric rearranged genome, TU/Arch$_N$, contained no DpnI-resistant DNA, indicating a lack of viral genome replication. Overall, these results suggest that the NCCR plays an important role in regulating BKV replication. Future studies will address whether the replication of the chimeric archetype genome, Arch/TU$_N$, is accompanied by expression of viral proteins and production of infectious virions. In addition, the remaining combinations of chimeric genomes will be constructed and analyzed for the ability to replicate in RPTE cells.

Based on these results, we hypothesized that TAg expression is a limiting factor for archetype BKV replication. The archetype early promoter has lower activity than rearranged early promoters and replacement of the archetype NCCR with the higher activity BKV TU NCCR induced viral DNA replication, suggesting that higher levels of TAg could rescue replication of archetype strains. To examine the effect of TAg expression on archetype replication, we cotransfected archetype or BKV TU genomic clones with a TAg expression plasmid. Low molecular weight DNA was harvested at 4

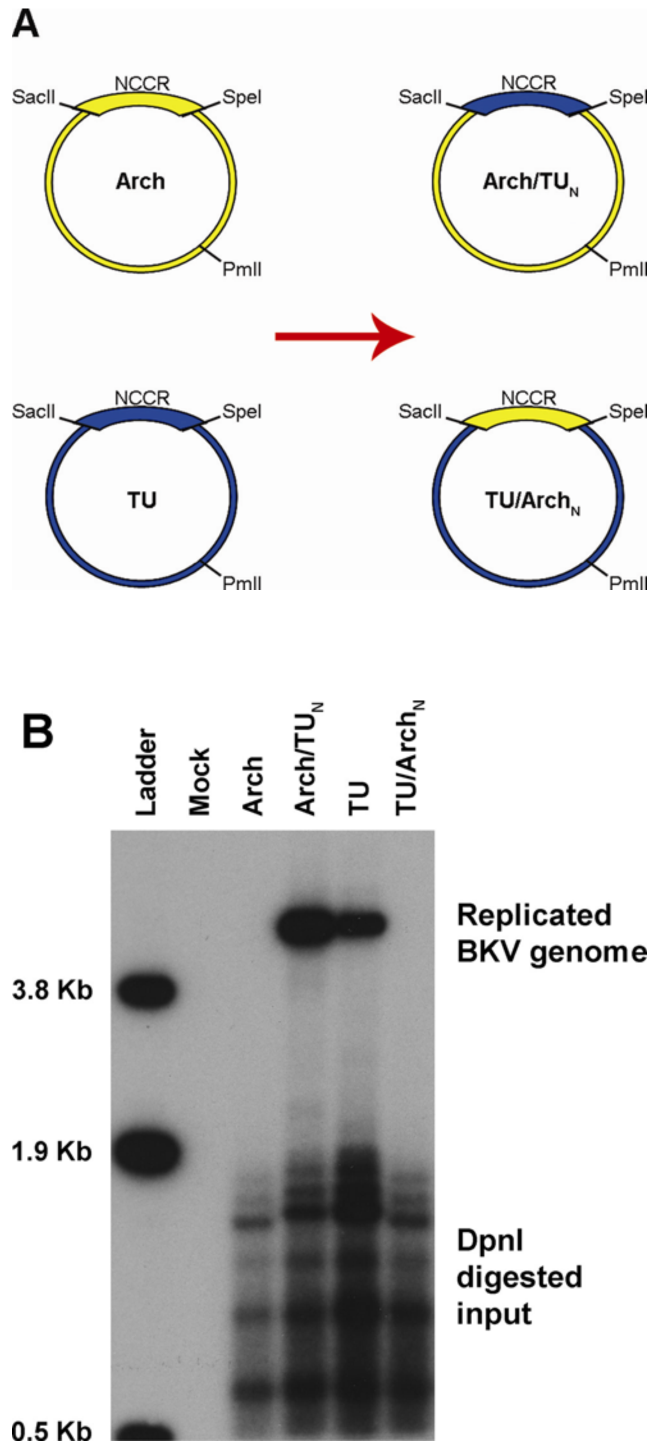

Figure 4.6. Rearranged NCCR can promote archetype BKV DNA replication. A) Cloning strategy for genomic region swaps. B) RPTE cells were transfected with the four plasmids from part (A). Low molecular weight DNA was harvested at 4 dpt and assayed by Southern blot for viral DNA replication, as described in Materials and Methods. Arch, archetype BKV; Arch/TU_N, archetype genome with TU NCCR; TU/$Arch_N$, TU genome with archetype NCCR; hpi, hours post-infection; dpt, days post-transfection; UN, untreated; Mock, no transfection.

and 7 dpt, digested with DpnI, and analyzed by Southern blot to detect replication of viral DNA (Figure 4.7). BKV TU DNA appears to replicate equally as well in the absence or presence of the TAg expression plasmid. The archetype genome, however, only replicates in the presence of TAg overexpression, indicating that elevated levels of TAg can drive archetype infection. The levels of replicated archetype DNA decreased at 7 dpt, most likely due to the limited duration of TAg expression with transient transfection. In addition, the diminished intensities of the DpnI digested bands at 7 dpt were likely a result of cell-mediated degradation of foreign DNA.

These results support the hypothesis that TAg levels are a limiting factor for archetype replication. In addition to providing insight into the requirements for productive BKV infection, these data will aide in the development of a tissue culture system to propagate archetype BKV strains. Future plans include using a cell line stably transformed with TAg to attempt to propagate and study archetype virus during a productive infection. Finally, our findings provide support for a model of BKV reactivation proposed in Chapter V (Figure 5.1), in which rearrangement of the NCCR precedes reactivation of archetype BKV. The rearranged NCCR is required to provide higher levels of TAg expression, which promotes the replication of archetype virus. These and future experiments will help us to better understand the process of archetype BKV persistence and reactivation in the human host.

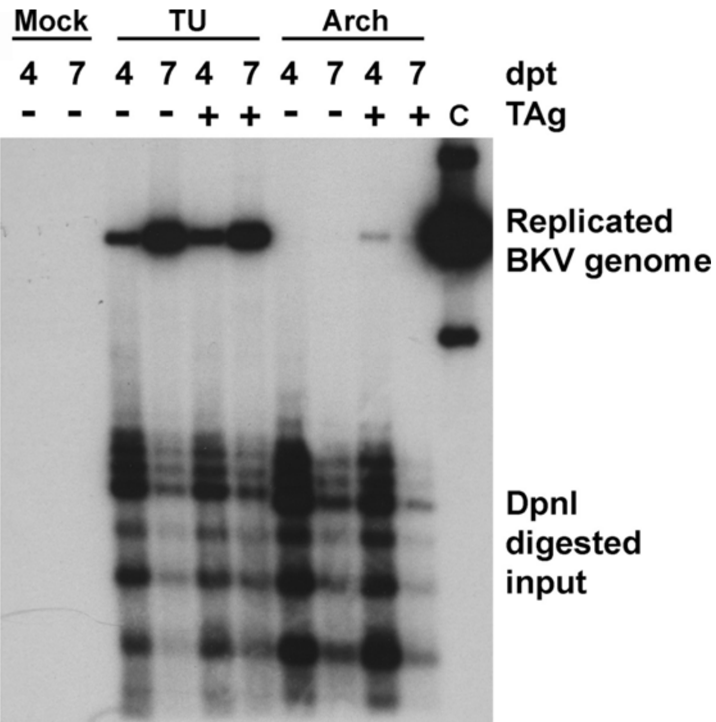

Figure 4.7. Ectopic expression of TAg can facilitate archetype BKV replication. RPTE cells were transfected with the rearranged (TU) or archetype (Arch) genomic clones, or cotransfected with a TAg expression plasmid. Low molecular weight DNA was harvested at 4 or 7 dpt and assayed by Southern blot for viral DNA replication, as described in Materials and Methods. Mock, no transfection; dpt, days post-transfection; C, control plasmid digested with EcoRI as a size marker.

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CHAPTER V

DISCUSSION

Summary of Results

BK virus (BKV) is a ubiquitous human pathogen, infecting nearly the entire population early in childhood and persisting throughout the lifetime of the host. In healthy individuals, BKV infection does not cause disease although viral shedding in the urine is evident periodically. BKV is widely accepted as the causative agent of polyomavirus nephropathy (PVN) in renal transplant recipients and late-onset hemorrhagic cystitis (HC) in bone marrow transplant recipients. The increasing prevalence of these BKV-associated diseases in immunosuppressed patients is likely a result of more potent immunosuppressive therapies, which may eliminate components of the immune system that are necessary to keep the virus in a subclinical state. We hypothesized that cytokines and cytokine-mediated signaling events are important for regulating BKV replication, and that the loss of cytokine-producing lymphocytes during immunosuppression allows BKV lytic infection to occur. This dissertation describes our investigation of the roles of two cytokines, IFN-γ and TGF-β, in regulating BKV gene expression and replication.

In Chapter II, after screening a panel of cytokines and chemokines, we identified IFN-γ as having a strong inhibitory effect on BKV early and late gene expression in a dose-dependent manner. In IFN-γ treated cells, we detected significant reductions in TAg transcripts at four days post-infection, suggesting that IFN-γ-mediated inhibition was occurring at the level of transcription. IFN-γ did not appear to change the kinetics of viral gene expression, but treatment of infected cells resulted in a significant decrease in viral progeny production. Finally, we demonstrated that the effect of IFN-γ was similar for three different strains of BKV, suggesting that this cytokine is relevant for the regulation of all BKV strains.

In Chapter III, we investigated the effect of TGF-β, a cytokine that is stimulated by certain immunosuppressive therapies, on BKV lytic infection. Viral gene expression, and specifically the activity of the BKV early promoter, was regulated by TGF-β in a strain-dependent manner. The TU strain of BKV had enhanced early promoter activity in the presence of TGF-β, while the Dik, Dunlop, and Proto-2 strains were either unaffected or had decreased promoter activity, depending on the cell type examined. Using site-directed mutagenesis, we identified a small segment of the TU promoter that is required for stimulation of activity in response to TGF-β. While these data suggest that TGF-β may play a role in BKV reactivation, perhaps more importantly the results demonstrate that BKV strains can respond differently to cytokine treatment.

We are primarily interested in the process of BKV reactivation in kidney epithelial cells, leading to the development of PVN in renal transplant recipients. The primary human renal proximal tubule epithelial (RPTE) cell culture system established by our lab (Low et al., 2004) is well suited to examine BKV replication in this context.

As discussed in Chapter I, we hypothesized based on clinical observations that cytokines regulate BKV during persistence in healthy individuals, and their differential expression in transplant patients facilitates reactivation. Our studies of IFN-γ- and TGF-β-mediated regulation are highly relevant and complementary. The primary targets of immunosuppressive agents are lymphocytes; most immunosuppression regimens include calcineurin inhibitors, antiproliferative agents, and/or corticosteriods to block the replication and activation of T cells and B cells. T cells, especially $CD4^+$ T helper 1 cells and $CD8^+$ cytotoxic T lymphocytes (CTLs), are major producers of IFN-γ and therefore levels of IFN-γ are reduced during immunosuppression. Concurrently, TGF-β expression is enhanced in renal proximal tubular epithelial cells by certain immunosuppressive drugs (Khanna et al., 1999a; Khanna et al., 1999b; McMorrow et al., 2005; Shihab et al., 1996). In addition, TGF-β is itself immunosuppressive and the signaling cascades of TGF-β and IFN-γ are antagonistic (Giannopoulou et al., 2006; Ulloa et al., 1999). Given our results that IFN-γ inhibits BKV replication and TGF-β enhances replication of certain BKV strains, we could hypothesize that immunosuppression creates a cytokine environment that is ideal for BKV reactivation.

Our observation that the TGF-β effect is strain-dependent, however, complicates this hypothesis. There are no data suggesting that specific rearranged strains are associated with enhanced pathogenesis, although NCCR rearrangements in general may be (Gosert et al., 2008). We were able to map the TGF-β-responsive elements in the TU strain; although we were unable to find any other strain with a predicted ZEB-1 binding site, a number of strains contain putative Smad3 binding sites (listed in Chapater III). Therefore, it is possible that additional TGF-β-responsive elements exist in other strains,

as Smad3 has many documented binding partners (Brown et al., 2007; Feng and Derynck, 2005). Furthermore, the effects of TGF-β are known to be different for epithelial cells, fibroblasts, and immune cells (Li et al., 2006; Rahimi and Leof, 2007); thus the use of these cell types in our assays may reveal other TGF-β regulated strains of BKV. In particular, likely candidates for such experiments include lymphocytes, which may play a role in the dissemination of BKV during primary infection (Doerries et al., 1994), and bladder epithelial cells, which are the sites of viral lytic replication during HC.

Finally, it is possible that the importance of TGF-β for BKV reactivation stems from the immunosuppressive and anti-inflammatory effects of this cytokine. Specifically, TGF-β inhibits the proliferation and differentiation of naïve T cells into effector cells, CTLs and T helper cells (Gorelik and Flavell, 2002; Li et al., 2006), thereby inhibiting expression of IFN-γ. In this scenario, the relevance of TGF-β signaling in BKV reactivation is indirect: a block in the production of IFN-γ would alleviate the repression of viral replication, but there is no direct regulation of promoter activity by TGF-β signaling components. In addition, TGF-β, in conjunction with IL-6, stimulates the development of T helper 17 cells, named for their ability to produce members of the IL-17 family of proinflammatory cytokines (Bettelli et al., 2008; Steinman, 2007; Tato and O'Shea, 2006). In particular, IL-17 has been implicated in immune-mediated tissue injury. Given that immunosuppression alone is not sufficient to cause PVN, and instead it appears that some renal tissue damage may also be required, IL-17 expression and signaling events may play a role in BKV reactivation and disease.

The best way to investigate hypotheses involving the regulation of immune cells and their effects is to use a small animal model for BKV persistence and reactivation.

Our lab has made several attempts to develop such a model. First, we tried to establish a mouse model of PVN using K virus, a murine polyomavirus that is genetically more similar to BKV than mouse polyomavirus (Py), most notably because it does not encode a middle T antigen (Imperiale and Major, 2007). Although previous reports describe the establishment of a persistent K virus infection in kidney epithelial cells of mice, we were unable to reproduce these results. Next, we collaborated with a lab interested in identifying the block to BKV replication in rodent cells, which may allow the subsequent development of a transgenic mouse that supports BKV lytic replication. This work has not yet revealed a cellular factor that confers permissiveness of rodent cells to productive BKV infection. Recently, a mouse model for PVN using Py was reported: mice underwent renal transplantation followed by infection with Py, and the characteristics and effects of viral replication were examined (Han Lee et al., 2006). Although this model demonstrated preferential Py replication in the graft resulting in accelerated graft failure, it did not incorporate a persistent infection prior to transplantation. In addition, the significant genetic differences between Py and BKV limit the usefulness of this system, as only questions about the host response to viral reactivation could be addressed. Establishment of an accurate small animal model for PVN would be extremely valuable for the study of BKV replication and the immune system during reactivation.

In Chapter IV, we described the preliminary results from two current areas of research. First, we have continued to investigate the regulation of BKV by IFN-γ-mediated signaling events, as this cytokine has inhibitory effects on all viral strains examined and thus is relevant for an overall understanding of the immune response to BKV. We demonstrate that IFN-γ does not affect viral trafficking or delivery of the

genome to the nucleus, which further supports regulation at the level of transcription. The inhibition appears to be long-lasting, effective to at least nine days post-infection, and is stronger if cells are treated with IFN-γ prior to infection. Pretreatment may cause the cells to adopt an antiviral state in which the factors that regulate BKV are activated and recruited to the nucleus even before infection; thus, when viral DNA enters the nucleus it is immediately repressed. We hypothesized that IFN-γ signaling results in a stable chromatin remodeling event that drives the viral genome into a closed conformation. Treatment of infected cells with broad spectrum histone deacetylase (HDAC) inhibitors restores viral gene expression and replication in the presence of IFN-γ, supporting this hypothesis.

The second area of research described in Chapter IV is aimed at identifying the factors required for archetype BKV strains to replicate in tissue culture. While we were optimistic that RPTE cells, the model system developed by our lab to mimic BKV lytic infection in the kidney, would support archetype replication, there was no indication of infectious progeny production or viral DNA replication in our assays. Instead, we began a systematic approach to identify the region of the archetype genome that is responsible for viral inactivity. By inserting unique restriction enzyme sites flanking the NCCR and the early coding region, we can exchange genomic fragments between the rearranged TU strain and the archetype Dik strain. Substitution of the rearranged NCCR into the archetype genomic clone promoted robust viral DNA replication. Furthermore, cotransfection of the archetype genome with a TAg expression plasmid also allowed replication of viral DNA. These findings suggest that the promoter activity of the NCCR determines the ability of a viral strain to replicate in tissue culture. We further

hypothesized that the NCCRs of rearranged and archetype strains may differ in chromatin structure, resulting in a difference in promoter activity. Thus, the preliminary results from both areas of research in Chapter IV will lead to a deeper investigation of chromatin remodeling events in the context of viral infection.

Major Questions in the Study of BKV Reactivation

There are at least four critical questions that must be addressed to further our understanding of BKV reactivation and associated disease in the context of immunosuppression:

1) What factors or conditions allow archetype BKV to replicate in the host but not in a tissue culture system?
2) What is the pathological relevance of the archetype and rearranged BKV strains?
3) Why are the archetype strains preferentially shed in the urine, while the rearranged strains are preferentially found in the blood?
4) Ultimately, what are the factors that drive BKV reactivation in kidney transplant patients?

We will discuss these questions in greater detail in the following paragraphs.

What factors or conditions allow archetype BKV to replicate in the host but not in a tissue culture system? As described in Chapters I and IV, there have been many studies demonstrating that archetype BKV strains do not replicate in tissue culture systems (Rinaldo et al., 2005; Rubinstein et al., 1991; Sundsfjord et al., 1994; Sundsfjord et al., 1990; Watanabe and Yoshiike, 1982; Watanabe and Yoshiike, 1986). In the human host,

however, these viruses replicate quite efficiently, since the vast majority of clinical isolates, both from healthy individuals and immunosuppressed patients, have archetype NCCR structures (Gosert et al., 2008; Markowitz et al., 1991; Negrini et al., 1991; Sharma et al., 2007; Sugimoto et al., 1989; Sundsfjord et al., 1999; Takasaka et al., 2004; ter Schegget et al., 1985). Based on these observations, it appears that archetype BKV strains are in no way defective at replication, and instead we can propose that there are either factors missing that may direct propagation or inhibitory factors present that are preventing it.

The simplest explanation for these observations is that archetype BKV is only able to productively infect very specific cell types in the host, and that these cells have not yet been used for propagation studies. Other viruses have demonstrated such specificity, notably human papillomaviruses, which replicate in stratified squamous epithelium in the host and rely on differentiation of the cells for progression through the viral life cycle (Howley and Lowy, 2007). It was not until the development of complex raft cultures that researchers were finally able to study and propagate papillomaviruses in a cell culture system (Asselineau and Pruniera, 1984; McCance et al., 1988). The restriction of archetype BKV replication is not related to entry or trafficking, since transfection of viral DNA does not allow propagation (see Chapter IV; Rinaldo et al., 2005; Rubinstein et al., 1991). Instead, this restriction is likely the result of differential expression of transcription factors that regulate viral early gene expression. Host cell restriction by transcription factor expression has been suggested for JC virus (JCV), for which productive infection of rearranged strains appears to be limited to cells that express high levels of NF-1/X (Messam et al., 2003; Monaco et al., 2001). Similarly, expression

of a specific transcription factor may also regulate archetype BKV replication, and the permissive cell types have simply not yet been examined.

One promising candidate cell type for susceptibility to archetype strains is primary bladder epithelial cells. BKV sequences have been detected in normal and neoplastic bladder epithelium (Monini et al., 1995). Furthermore, the association of BKV with HC, a bladder infection, and the prevalence of virus in urine samples from healthy and immunosuppressed individuals suggest susceptibility of these cells to infection. We are currently investigating whether primary bladder epithelial cells will support productive archetype BKV infection. However, the bladder and ureter are lined with transitional epithelium, layers of epithelial cells not unlike stratified squamous epithelium, which allow the bladder to contract and expand. It is possible that archetype BKV will require a more complicated cell culture system, like that of human papillomavirus, to efficiently replicate.

Alternatively, it is possible and perhaps more likely that archetype strains require a positive factor to initiate replication, one that persistently infected cells are exposed to only during certain conditions. For example, it has been shown that the S block of the NCCR contains steroid hormone response elements and treatment of BKV-infected cells with glucocorticoids, estrogen, or progesterone results in higher viral yields (Moens et al., 1994). A hormone-dependent enhancement of viral replication would be relevant for reactivation in kidney and bone marrow transplant patients, who are often treated with corticosteroids as a part of their immunosuppressive regimen. There is some evidence suggesting that corticosteroid therapies are associated with an increased risk for BKV reactivation and PVN (Hirsch et al., 2002; Trofe et al., 2003). In addition, progesterone-

mediated enhancement of viral replication may be relevant to regulation of BKV during pregnancy. Viral shedding is more prevalent in pregnant women compared to the general population, particularly in the second and third trimesters when the levels of progesterone peak (Bendiksen et al., 2000; Boldorini et al., 2008; Coleman et al., 1980; Markowitz et al., 1991). Thus, steriod hormones may be positive factors that stimulate cells to be permissive to archetype BKV replication.

Another possible factor required for propagation of archetype strains may be elevated levels of TAg expression. As previously discussed (Chapter I and Chapter IV), archetype strains are known to have lower early promoter activity than rearranged strains. It is possible that these viruses do not produce enough TAg to facilitate viral DNA replication. JCV archetype and rearranged strains have characteristics very similar to those described for BKV (reviewed in Yogo and Sugimoto, 2001). Propagation of JCV archetype strains has been demonstrated in cells ectopically expressing JCV TAg (Sock et al., 1996) and COS-7 cells, which stably express SV40 TAg (Hara et al., 1998), without the induction of NCCR rearrangements. Based on our preliminary results in Chapter IV, it is likely that the same will be true for archetype BKV, however, further studies are needed to demonstrate progeny production and stability of the NCCR.

As proposed in Chapter IV, there may be differences in viral chromatin structure that could explain the ability of rearranged strains to replicate more efficiently than archetype strains. For example, the archetype NCCR may contain more or higher affinity binding sites for transcription factors that recruit HDACs, leading to condensation of the genome and restriction of transcription and replication. Conversely, rearranged NCCRs may have additional binding sites that aide in the recruitment of histone

acetyltransferases (HATs), leading to an open chromatin structure that promotes transcription and replication. In either case, the positive factor required for archetype replication could be one that inhibits HDAC (or stimulates HAT) activity or recruitment. Many signaling cascades, including those activated in response to cytokines, could be responsible for such an effect by activating or inducing the appropriate transcription factors. However, treating cells with HDAC inhibitors may allow efficient propagation of archetype virus, and the subsequent study of BKV minichromosome structures and factors that participate in regulation under different conditions.

What is the pathological relevance of the archetype and rearranged BKV strains?

For JCV, the pathological relevance of NCCR structure is quite clear. JCV is the causative agent of progressive multifocal leukoencephalopathy (PML), a fatal demyelinating disease that affects severely immunosuppressed individuals (reviewed in Khalili et al., 2006). Viral genomes containing promoter regions with a conserved linear structure, designated archetype, are primarily isolated from urine samples, kidney, and tonsillar tissues of healthy individuals (Agostini et al., 1996; Jeong et al., 2004; Kato et al., 2004; Tominaga et al., 1992; Yogo et al., 1990). Viral genomes with NCCR rearrangements, deletions, or amplifications are isolated routinely from the brains of PML patients and are designated PML-type strains (Loeber and Doerries, 1988; Martin et al., 1985). Furthermore, PML-type strains only replicate efficiently in human glial cells, the site of lytic infection and disease, and stromal cells and B lymphocytes, which likely aide in viral dissemination to the brain (Imperiale and Major, 2007). Thus, NCCR

rearrangements are highly associated with JCV lytic infection in the brain and progression to PML.

Based on the similarities between BKV and JCV, we would predict that NCCR rearrangements in BKV NCCRs would also correlate with disease progression. However, the fact that BKV both causes disease and maintains persistence at the same sites in the host, the kidney and bladder epithelium, creates difficulty in distinguishing strains associated with disease from those associated with persistence and transmission. In a recent report, Gosert *et al.* (2008) made several important observations. First, rearranged strains were more frequently detected in the plasma than in the urine, although archetype strains still predominated in both. Second, patients with rearranged BKV had significantly higher viral loads in the blood. Finally, among patients that had progressed to PVN, those with rearranged BKV had more inflammation and more extensive cell damage than patients with archetype BKV, as determined by examination of kidney biopsies. This is the first study to correlate BKV NCCR rearrangement with more severe disease in kidney transplant patients.

While it is possible that certain conditions of the host could predispose archetype BKV to acquire NCCR rearrangements, it seems likely that the changes occur randomly and sporadically, especially since rearrangements have been inconsistently observed in parallel cultures *in vitro* (Rinaldo et al., 2005). The functional relevance of the rearrangement might be very general: the resulting viral promoter is more active for early region transcription, resulting in more DNA replication and progeny production. The higher viral loads then lead to greater cell damage and more severe disease. In immunocompetent individuals, robust expression of viral antigens could lead to early

detection by the immune response and elimination of virus-infected cells before progeny production. Thus, in healthy individuals, rearranged strains may be at a disadvantage due to activation of the immune response, while archetype strains may be more adept at immune avoidance, and thus persistence and replication.

In contrast, it is possible that NCCR rearrangements change the cell tropism of the virus. This would explain the difficulty in propagating the archetype strains in the same cells that support replication of rearranged strains. Alternatively, NCCR rearrangements might change the way the virus is regulated in response to cytokine-mediated signaling or other factors. As suggested in Chapter III, the presence or absence of transcription factor binding sites can result in differential regulation of viral promoters in the presence of cytokines. There is one report suggesting that BKV strains containing a mutated Sp1 binding site are associated with development of hemorrhagic cystitis in bone marrow transplant recipients (Priftakis et al., 2001). Gosert *et al.* (2008), however, did not report any NCCR rearrangements or binding sites that were common among the isolates from the blood of PVN patients. While differential regulation by cytokines is an appealing idea, it is not yet supported by clinical data.

It is important to restate that the majority of BKV sequences in clinical samples have archetype NCCR structures. Gosert *et al.* (2008) report that 85 and 92% of sequences in the blood and urine, respectively, were archetype BKV and that approximately 50% of the patients that developed PVN were infected with archetype strains. These data argue against a clinical relevance for NCCR rearrangements, as it is clear that the replication of archetype strains can result in disease. Instead, perhaps rearranged NCCRs facilitate the replication of archetype strains by providing higher

levels of TAg expression in *trans* (as discussed above). If NCCR rearrangements are random events, it should be possible to have genomes with changes in the coding regions as well. Thus, in addition to the functional NCCR rearranged strains, there could also be non-functional genomes with coding region rearrangements that disrupt viral gene expression and are defective for replication. One could then envision a population of viral genomes with highly active rearranged NCCRs and disruptions to the late coding region. These genomes would express high levels of TAg but no late proteins, and therefore would be defective for replication themselves; however, the expression of TAg from these 'helper' viruses could facilitate archetype BKV replication. In this scenario, rearrangements in the NCCR may be indirectly essential for archetype BKV persistence and reactivation.

Why are the archetype strains preferentially shed in the urine, while the rearranged strains are preferentially found in the blood? This question is perhaps a bit misleading: archetype strains of BKV are actually the most common strains found in both blood and urine samples, as described above and in Chapter I. Interestingly, Gosert *et al.* (2008) observed that the viral load of archetype BKV in the urine was significantly higher than that of rearranged BKV, while the opposite was true in the blood. We can propose two different explanations for this observation: either the two types of virus are replicating in different cells or the progeny are being released in different ways.

Based on the inability to propagate archetype strains in cells that are highly susceptible to infection with rearranged BKV, it is possible that the two types of virus exhibit different cell tropisms (this idea has been discussed in the sections above). It is

necessary to assume, however, that archetype BKV can readily infect kidney epithelial cells, although it may require specific conditions to productively replicate. Archetype strains are thought to be the transmitted form of BKV, and the derivation of rearranged strains from archetype genomes has been demonstrated. Therefore, archetype virus must be capable of establishing persistence in the kidney. If archetype strains are also able to infect additional cell types, such as primary bladder epithelial cells, then simultaneous infection of kidney and bladder epithelial cells could allow archetype progeny to be released into the blood and urine. If rearranged strains can only infect the kidney epithelium, then viral progeny may be channeled primarily into the blood.

The alternative explanation is that there is a difference in the release of archetype and rearranged progeny from the cell. Very little is known about how polyomaviruses exit the infected cell, but there are reports describing both lytic bursts and viral egress from intact cells (Imperiale and Major, 2007). Although there has been no direct comparison made between the replication kinetics of archetype and rearranged virus in the host, the behavior of these two viruses in tissue culture suggests that archetype BKV has a slow replication cycle and rearranged strains replicate quickly and robustly. Perhaps this rapid production of rearranged BKV progeny overwhelms the normal viral egress pathway and instead virions are released by a lytic burst. The destruction of epithelial cells may help the virus spread and infect surrounding cells, including the endothelial cells of nearby blood vessels. Replication in and subsequent lysis of these cells could release the virus into the bloodstream. In contrast, the slower replication of archetype strains could promote viral egress, leaving infected cells intact and perhaps favoring release of virus in the urine instead of the bloodstream. The presence of

archetype virus in the blood of immunocompromised patients with PVN may indicate that extensive and unregulated replication is also possible with archetype strains and will result in cell lysis.

Differences in viral release could also be mediated by mechanisms of egress. Epithelial cells are polarized and thus have apical surfaces, which face the lumen, and basolateral surfaces, which contact the surrounding epithelial cells. Many viruses have been shown to release progeny preferentially from one of these surfaces, including hepatitis A (Blank et al., 2000), measles virus (Blau and Compans, 1995), SV40 (Clayson et al., 1989), and Epstein-Barr virus, for which preferential basolateral release has been suggested to favor viral dissemination (Chodosh et al., 2000). Rearranged strains may somehow be defective for viral egress at the apical surface but efficient at release from the basolateral surfaces, leading to dissemination into the blood. In contrast, archetype strains may either mediate apical release preferentially from bladder epithelial cells and basolateral release from kidney epithelial cells, or perhaps have no preference for release. NCCR rearrangements could affect viral release by regulating replication kinetics, as described above. Alternatively, rearrangements could result in differential regulation of agnoprotein expression; very little known about this protein, but it is suggested to be involved in virus maturation and release (Rinaldo et al., 1998).

Ultimately, what are the factors that drive BKV reactivation in kidney transplant patients? Currently, the list of potential risk factors for PVN includes older age, male gender, seropositivity of the donor, seronegativity of the recipient, specific immunosuppressive drugs, HLA mismatches, lack of the HLA-C7 allele in the donor or

recipient, acute rejection episodes prior to development of PVN, and low numbers of BKV-specific IFN-γ-producing T cells (Comoli et al., 2006; Egli et al., 2007). Immunosuppression regimens target lymphocytes, the major IFN-γ-producing cells, and prevent the activation and proliferation of these cells. In Chapter II, we describe the inhibitory effect of IFN-γ on BKV replication. A reduction in levels of this cytokine is likely a key factor that allows BKV reactivation. In Chapter IV, we discuss future research plans to examine the specific factors involved in this regulation. Transcription factor binding site prediction programs were of limited help in identifying potential responsive elements since IFN-γ-mediated inhibition of viral replication was strongest at late stages of infection, when the effects of IFN-γ signaling are robust and widespread, as a result of the activation and induction of many signaling components.

While IFN-γ is a well-known antiviral cytokine, it is not frequently shown to have direct inhibitory activities on viral gene expression. It is far more common for the type I interferon signaling pathway to mediate direct effects on viral replication, while IFN-γ signaling typically regulates the cellular immune response and activates cytotoxic T lymphocytes to kill virus-infected cells. In our studies, IFN-α did not significantly affect BKV gene expression, suggesting that the virus is able to somehow avoid effectors of the type I pathway. It is possible that transcription factors induced or activated by IFN-α/β signaling may be unable to regulate the BKV promoter due to a lack of the appropriate binding sites. Alternatively, expression of an early viral protein, likely TAg based on similar findings for SV40 and Py (Swaminathan et al., 1996; Weihua et al., 1998), may block the IFN-α/β signaling pathway. This explanation correlates with our observations that BKV infection does not activate any innate immune response in kidney epithelial

cells (J. Abend, J. Low, and M. Imperiale, unpublished data), and may also partially explain how BKV establishes persistence.

We hypothesize that a key factor mediating reactivation is intense immunosuppresion of transplant patients. The immunosuppressive regimen during the first year post-renal transplant is typically at higher doses than during later years post-transplant. Thus, it is possible that patients receive more immunosuppressant than is necessary to maintain the balance between preventing graft rejection and controlling viral reactivation. In addition, there may be a subset of transplant patients that naturally have lower levels of cytokine production, and therefore would be more sensitive to immunosuppression. Pravica *et al.* (1999) identified a polymorphism in the IFN-γ promoter that results in higher levels of IFN-γ production. The polymorphism relates to the number of consecutive CA repeats, and the allele (12 CA repeats, designated allele 2) confers high production of IFN-γ in either homozygous or heterozygous individuals. Allele 2 is more frequent than any other allele, with 75% of the population having at least one copy (Pravica et al., 1999). Thus, 25% of the population is negative for allele 2 and consequently has approximately two-fold lower levels of IFN-γ production, based on stimulation of PBMCs *in vitro*. While this may not sound impressive, the effect *in vivo* and in the presence of immunosuppressive drugs may be more dramatic. The frequency of low IFN-γ producers seems to roughly correlate with the frequency of PVN and HC: if 25% of the transplant population is negative for allele 2, then it is reasonable to expect about half of these patients (10%) have another risk factor, such as age gender or donor seropositivity, and develop PVN.

In addition to polymorphisms that alter IFN-γ production, there are also polymorphisms that determine production levels of other relevant cytokines, for example, TGF-β (Grainger et al., 1999). The IL-12 gene has a single nucleotide polymorphism that reduces the production of the p40 subunit of IL-12 (Stanilova and Miteva, 2005), and has recently been reported as a risk factor for human cytomegalovirus infection after kidney transplantation (Hoffmann et al., 2008). IL-18 also has promoter polymorphisms that confer levels of expression (Giedraitis et al., 2001) and are implicated in disease outcome. IL-12 and IL-18 (also known as the IFN-γ-inducing factor) are the major activators of IFN-γ production, and can act alone or in synergy. Polymorphisms that confer low levels of expression of these cytokines could be associated with a higher risk of BKV reactivation. It would be interesting and useful to determine if there is a correlation between these and other polymorphisms, and progression to HC or PVN. Patients could easily be screened for such polymorphisms prior to transplantation to determine if there is a high risk for reactivation. In this situation, the immunosuppressive regimens could be adjusted and monitored.

Although immunosuppression is important, the infrequency of BKV-associated disease in non-renal solid organ transplant recipients and patients with AIDS indicates that there are other conditions required for reactivation. One such kidney-specific risk factor could be damage to the graft as a result of ischemia and reperfusion during transplantation. Ischemia/reperfusion events may activate factors within BKV-infected cells that could promote viral reactivation, and since the kidney is the major site of BKV persistence, these events would only affect renal transplant patients.

Conclusions

Based on the discussions in this chapter, a proposed model for the replication of archetype virus (left) and the factors that determine the prevalence of rearranged virus in the blood (right) is depicted in Figure 5.1. First, the archetype virus establishes a persistent infection in kidney epithelial cells but is only able to express very low levels of TAg that do not support replication. Random rearrangements produce a viral genome that has a highly active early region promoter but is defective for late gene expression, either by disruption of the coding region or an essential element of the late region promoter. The resulting high levels of TAg then promote replication of archetype virus without producing rearranged virus. The second part of this figure shows archetype virus release by a cell-mediated egress pathway, which leaves the infected cell intact. The slower kinetics of archetype replication in healthy individuals may promote the use of this pathway, and egress may be directed to the apical side of the epithelial cell resulting in preferential release in urine. The faster, more robust replication of rearranged strains may prevent utilization of this pathway, and instead result in a viral lytic burst. Destruction of the infected cell may promote spread to surrounding cells, in particular vascular endothelium, resulting in a preference for the release of rearranged strains into the bloodstream. It is likely that archetype strains also cause cell lysis under certain conditions of robust replication, as these viruses are found both in blood and urine.

In conclusion, this dissertation has provided an overview of BKV biology and pathogenesis, and a description of our findings on cytokine-mediated regulation of viral gene expression and replication. We have also shown preliminary data on the mechanism of IFN-γ-mediated regulation and the replication of archetype strains in culture, and

proposed further studies. Finally, we discussed the major questions in the field of BKV reactivation in transplant patients. In the absence of effective antiviral therapies for BKV, a better understanding of persistence and factors that drive reactivation will provide insight into better options for the treatment and prevention of PVN and HC.

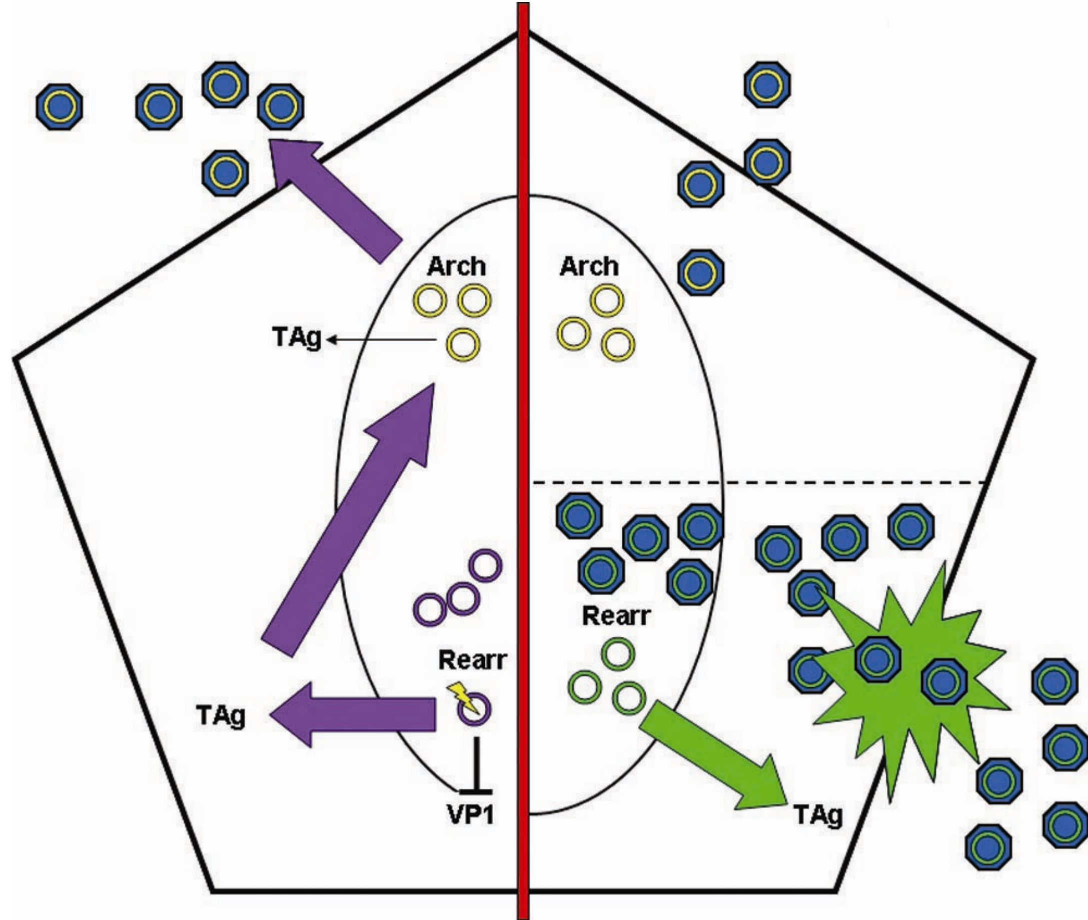

Figure 5.1. Proposed model for replication of archetype virus and factors that determine the prevalence of rearranged virus in the blood. Replication of archetype virus (left): archetype virus persistence is established in a cell (genomes shown by yellow circles). Weak TAg expression (thin arrow) does not support replication, however, random rearrangements produce a viral genome (purple circle) that has robust early promoter activity but is unable to produce VP1 and thus unable to propagate. The high levels of TAg (thick purple arrow) are utilized by the archetype virus to facilitate replication. Virus release pathways (right): archetype virus utilizes a cell-mediated egress pathway for progeny release, leaving the infected cell intact. Rearranged virus (genomes shown by green circles), replicates robustly and overwhelms the cell with progeny, leading to a viral lytic burst (green star shape). Arch, archetype; rearr, rearranged.

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