

DETERMINANTS OF ARCHETYPE BK POLYOMAVIRUS REPLICATION

by

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Abstract

BK polyomavirus (BKPyV) is a small double-stranded DNA virus that is an emerging pathogen in immunocompromised individuals. BKPyV infection is widespread in the general population. Following an initial asymptomatic infection, it establishes persistence in the urinary tract of its human host. BKPyV can reactivate and cause diseases such as polyomavirus-associated nephropathy and hemorrhagic cystitis under conditions of immune suppression in renal and bone marrow transplant recipients, respectively.

Previous research in our laboratory and others has focused on the host factors that contribute to controlling lytic replication of BKPyV. The focus of this dissertation is to better understand viral mechanisms of regulation that control replication, specifically how the virus is able to establish and maintain a state of persistence following initial infection. Therefore we set out to characterize the transmissible form of BKPyV, archetype virus, which is thought to establish persistence in the host. This virus is defined as having a linear O-P-Q-R-S arrangement of sequence blocks in the non-coding control region (NCCR), of the genomic DNA, as opposed to rearranged variants which have deletions and duplications in their blocks. Rearranged variants can arise during reactivation and are found almost exclusively in patients with BKPyV-associated diseases. Our laboratory developed a natural cell culture model of rearranged variant replication in primary renal proximal tubule epithelial (RPTE) cells. However, archetype virus does not produce progeny in RPTE cells. We determined that of the three major genetic regions that make up the BKPyV genome, the early coding region, the late coding region, and the

NCCR, the NCCR is responsible for the replication phenotype that differentiates archetype and rearranged virus in RPTE cells. However, we can artificially induce archetype virus propagation in 293TT cells, which overexpress large T antigen, a protein that is required for DNA replication. Furthermore, archetype virus uniquely displays differential promoter activity, driving high levels of a regulatory miRNA early, prior to DNA replication. This miRNA targets early mRNA, thereby limiting virus replication in RPTE cells. This means of repressing viral replication likely contributes to the mechanism of archetype virus persistence in the host.

Since there is currently no model of persistence for any human polyomavirus, we have begun to directly examine archetype BKPyV persistence in a cell culture model of urothelial cells. Urothelial cells are a transitional epithelium in the bladder, ureter, and pelvic wall with direct access to the urine, the most common site of archetype virus isolation. These cells are a promising candidate to examine maintenance of the archetype virus genome and test potential reactivation stimuli. Overall, our results advance our current understanding of archetype polyomavirus replication and persistence.

Chapter I

Introduction

BK polyomavirus

BK polyomavirus (BKPyV) was one of the first two human polyomaviruses identified. BKPyV and JC polyomavirus (JCPyV) were isolated from immunosuppressed patients and were described in back-to-back articles in *Lancet* in 1971 (Gardner et al., 1971; Padgett et al., 1971). BKPyV was named for the initials of the patient from whom it was originally isolated, a renal transplant patient with ureteric stenosis. BKPyV, JCPyV, and the closely related simian virus 40 (SV40) were originally grouped with papillomaviruses in the genus *Papovavirus*, which was later separated into the genera *Papillomavirus* and *Polyomavirus*. Recently, the International Committee on Taxonomy of Viruses has recommended that the naming of all polyomaviruses be standardized (Johne et al., 2011). Following taxonomical revisions, it was recommended that the family *Polyomaviridae* contain two mammalian genera designated *Orthopolyomavirus* and *Wukipolyomavirus* (Johne et al., 2011). In addition to BKPyV and JCPyV, the *Orthopolyomavirus* genus includes the more recently discovered human viruses Merkel cell polyomavirus (MCPyV) and trichodysplasia spinulosa-associated polyomavirus (TSPyV) (Feng et al., 2008; van der Meijden et al., 2010). KI polyomavirus, WU polyomavirus, Human polyomavirus-6, -7, -9 comprise the *Wukipolyomavirus* genera (Allander et al., 2007; Gaynor et al., 2007; Schowalter et al., 2010; Scuda et al., 2011). Recently, however, new polyomaviruses:

HPyV10, MWPyV, MXPpyV, and STLPyV have been identified that do not fit the current taxonomical groupings (Buck et al., 2012; Lim et al., 2013; Siebrasse et al., 2012; Yu et al., 2012).

Polyomaviruses are small double-stranded DNA viruses that are non-enveloped and cause persistent infections. This group of viruses can cause a variety of diseases depending upon the site of lytic reactivation. For example, in humans, JCPyV reactivates in the brain, causing progressive multifocal leukoencephalopathy, and MCPyV causes Merkel cell carcinoma in the skin. BKPyV reactivation leads to polyomavirus-associated nephropathy (PVAN) in the kidney or hemorrhagic cystitis (HC) in the bladder.

The BKPyV particle consists mainly of three molecules that serve as the building blocks for the structure of the viral capsid: the major structural protein VP1 and less abundant minor capsid proteins VP2 and VP3. The capsid surrounds the double stranded circular DNA genome that is complexed with histones in the form of a miniature chromosome. The BKPyV genome is divided into three major genetic regions: the early coding region, the late coding region, and the non-coding control region (NCCR) (Imperiale et al., 2007a). There are two different forms of the virus, termed archetype virus and rearranged variants, based on the DNA sequence structure of the NCCR. Archetype virus can be commonly isolated from the urine of both healthy people as a result of transient reactivation events, and diseased patients (Doerries, 2006). In contrast, rearranged variants are most often found in the serum of patients with BKPyV-associated diseases.

While upwards of 90% of the population is persistently infected with BKPyV, the virus reactivates and is asymptotically shed into the urine in 5-10% of immunocompetent adults at any given time (Knowles, 2001). Additionally, BKPyV viruria can be detected in up to 25% of

pregnant women (Jin et al., 1993a). BKPyV is a significant human pathogen that causes disease in immunocompromised individuals. BKPyV is able to reactivate in renal transplant and bone marrow transplant (BMT) patients in whom a lytic infection results in PVAN and HC, respectively. Rarely, BKPyV reactivation and disease have been observed in other immunocompromised conditions such as systemic lupus erythematosus, in other solid organ transplant recipients, and in patients with HIV/AIDS (Jiang et al., 2009a).

BKPyV is an emerging pathogen whose incidence continues to increase as a result of the growing number of transplant patients and the development of better immunosuppressive drugs. Unlike JCPyV, which was recognized early on as an opportunistic pathogen as a result of the HIV/AIDS epidemic, BKPyV was not acknowledged to be a pathogen until more recently. Historically, polyomaviruses, especially SV40, have been utilized to advance our understanding of basic eukaryotic cellular processes but have been less well studied for their significance as human pathogens. In this Chapter, I describe what is currently known of BKPyV infection both at the level of the affected patient and at the level of the infected cell.

Viral persistence

Polyomaviruses establish a persistent viral infection that last for the life of the host. It is unclear if polyomavirus infection of a cell establishes a general persistent infection with minimal maintenance of the viral genome or if it can establish a state of viral latency. Viral latency is a type of viral persistence that is defined by complete genome maintenance, restricted antigen expression, lack of progeny production, and reversibility (Speck et al., 2010). Reversibility, the capacity for full viral reactivation or lytic infection, is the key defining characteristic of viral latency. In this form, the virus can avoid detection by the host immune system by limiting

production of viral antigens. Few viruses undergo true latency in the host; however, the mechanisms of viral latency are best studied in the herpesviruses. During herpesvirus latency, transcription is limited to genes whose functions include directing episomal replication and segregation, modulating host signaling, and suppressing the regulator of lytic reactivation. Importantly, herpesvirus microRNAs (miRNAs) are significant regulators of genes controlling viral lytic replication (Grey et al., 2007; Murphy et al., 2008). The discovery, function, and possible role in persistence of the polyomavirus miRNA will be discussed later.

Very little is known about the mechanism of polyomavirus persistence since there is no cell culture model for any archetype human polyomavirus. However, the life cycle and persistence of human papillomavirus (HPV), which is a closely related virus to polyomavirus, is well studied in a stratified culture model in keratinocytes (Moody et al., 2010). HPV infects, and is episomally maintained in, the keratinocyte basal layer. Viral progeny production is induced upon cellular differentiation of infected cells, and virions are released from the upper layer of the epithelium. Many polyomaviruses including MCPyV, TSPyV, human polyomaviruses-6, -7, -9, and -10 have been recently isolated from skin, a stratified cell system. This supports the hypothesis that polyomaviruses may also persist in a stratified cell culture model and reactivate as these cells differentiate. Unlike herpesviruses and polyomaviruses, HPV does not encode any viral miRNAs. Therefore, the mechanism of polyomavirus persistence may combine features of HPV persistence in stratified cells and autoregulation of herpesvirus latency. These concepts and a preliminary cell culture model of archetype BKPyV persistence will be discussed in Chapter V.

Transmission and epidemiology

BKPyV transmission is thought to occur via a respiratory route, based on evidence of high seroconversion rates in the population by mid-childhood, with 65-90% of humans becoming seropositive by the age of ten, and on the presence of viral DNA in tonsillar tissue (Goudsmit et al., 1982; Knowles, 2006). There is also evidence for other possible routes of transmission such as fecal-oral, urino-oral, blood transfusion, and transplacental (Hirsch et al., 2003; Jiang et al., 2009a; Vanchiere et al., 2005b). Archetype virus is thought to be the transmissible form of BKPyV because its NCCR structure can give rise to all known rearrangements and because it is isolated from both healthy people and diseased patients. Primary infection is thought to be asymptomatic or result in a mild respiratory illness (Goudsmit et al., 1982). BKPyV then disseminates to the urinary tract where it persists for the life of the host (Heritage et al., 1981a). Immunostaining has shown the main sites of BKPyV replication to be in epithelial cells of the kidney, ureter, and bladder (Shinohara et al., 1993). BKPyV DNA has been detected in peripheral blood mononuclear cells, which suggests that BKPyV may hijack the immune system to spread from the primary site of infection to its site of persistence (Chatterjee et al., 2000). It is unclear if the virus persists with minimal maintenance replication of its genome or if it enters a true state of viral latency. Periodically, BKPyV can reactivate and be shed into the urine of healthy hosts, which contributes to the evidence of a possible urino-oral infectious route (Doerries, 2006).

Reactivation and clinical disease

BKPyV is an opportunistic pathogen that primarily causes disease in transplant patients. HC in BMT recipients is a condition associated with high morbidity and is characterized by

symptoms including dysuria, frequency, urgency, subrapubic pain, and varying degrees of hematuria (Dropulic et al., 2008). A grading system to assess the severity of HC was developed by Droller *et al.*, where grade 1 is microscopic hematuria; grade 2, visible hematuria; grade 3, visible hematuria with small clots; and grade 4, gross hematuria with urinary tract obstruction caused by clots (Droller et al., 1982). Mild cases of HC will usually resolve within two weeks with supportive care. In more serious cases, significant bleeding and clot formation can cause urinary tract obstruction, severe pain, uncontrollable bleeding, and acute renal failure. In rare instances, fatality may occur as a result of HC.

In BMT patients, HC can occur both pre- and post-engraftment but is attributed to different causes, chemotherapeutic agents or viral infection, respectively. Pre-engraftment HC occurs from the direct cytotoxic effects of antineoplastic treatments, generally a toxic build up of acrolein, a metabolite byproduct of the processing of cyclophosphamide or ifosfamide (Cox, 1979). However, there is now an effective treatment of forced diuresis that has eliminated virtually all chemotherapeutic-induced HC. Cytomegalovirus (CMV), adenovirus, and BKPyV are able to cause HC post-engraftment, for which BKPyV causes the majority.

BKPyV-associated HC occurs in 10-25% of BMT patients and typically presents two weeks post-transplantation (Dropulic et al., 2008). However, BKPyV viruria can be detected in 50% of BMT patients. High levels of BKPyV viruria may be associated with increased risk of HC development. One study also found that plasma viral loads greater than 10^4 copies/ml were associated with increased risk of HC development (Erard et al., 2005).

Diagnosis of BKPyV-associated HC is often made by cytological examination of the urine or by PCR amplification of viral DNA in the urine or serum. Characteristic infected cells, called decoy cells, can be observed in the urine of affected patients. These cells have enlarged

nuclei and one large basophilic intranuclear inclusion. However these cells are not specific for BKPyV infection, since adenovirus can cause similar cytopathology. Therefore, PCR amplification of BKPyV viral DNA from the urine or the serum is more specific and is used commonly to diagnose BKPyV-associated disease in the presence of HC symptoms. BKPyV viral loads in the urine of HC patients can peak as high as 10^{14} copies/ml (Wong et al., 2007). The current standard treatment protocol for BKPyV-associated HC is supportive care including pain management, hyperhydration, forced diuresis, and bladder irrigation (Dropulic et al., 2008).

PVAN is characterized by an inflammatory interstitial nephropathy that presents on average 10 to 13 months post renal transplant (Boothpur et al., 2010). PVAN most commonly occurs in the graft kidney of renal transplant patients and is rare in the native kidney of other solid organ transplant recipients. This is likely due to the level of immunosuppression and injury at the site of viral persistence, the kidney, rather than general immunosuppression alone. The development of better immunosuppressive drugs in the last two decades has led to a decreased rate of acute rejection in renal transplant patients but an increase in the incidence of PVAN (Ramos et al., 2009). PVAN occurs in up to 10% of kidney transplant patients, up to 50% of whom will lose their graft (Ramos et al., 2009).

The immunosuppressive regimen for renal transplant patients varies somewhat but commonly consists of a calcineurin inhibitor, an antimetabolite, and a corticosteroid. Calcineurin inhibitors such as cyclosporine and tacrolimus inhibit immunophilins and reduce the transcription of IL-2, which has the overall effect of reducing T cell activation. Antimetabolites such as azathioprine and mycophenolate mofetil inhibit nucleic acid synthesis and cell proliferation, especially in lymphocytes. Corticosteroids are anti-inflammatory and have other

immunoregulatory properties. Together these drugs reduce the host immune response to avoid graft rejection but unfortunately contribute to viral reactivation.

There is no single risk factor strongly associated with the development of PVAN in renal transplant recipients. Rather, a variety of weakly associated risk factors have been identified for BKPyV reactivation. Pre-transplantation risk factors can be divided into recipient and graft characteristics. Recipient characteristic risk factors include: older age, male sex, seronegativity, and previous graft loss caused by PVAN. Graft characteristic risk factors include: seropositive donor, degree of HLA mismatch, and ischemic or immunological injury. The immunosuppressive regimen is the major post-transplantation factor identified in virus reactivation (Suwelack et al., 2012). It is thought that the degree of immunosuppression, and not the specific therapy, contributes to PVAN development. For instance, the incidence of PVAN increased with the advent of more potent immunosuppressants such as tacrolimus and mycophenolic acid (Binet et al., 1999; Ramos et al., 2009). In addition, there are viral factors that may increase the relative risk of BKPyV reactivation. For example, mutations within the BKPyV VP1 capsid protein are associated with increased pathogenesis (Randhawa et al., 2002). Also, it was recently reported that BKPyV genotype IV is a unique serotype from genotype I and that although 95% of renal transplant recipients have antibodies with neutralizing activity for genotype I (Pastrana et al., 2012). This neutralizing activity does not protect against genotype IV BKPyV, for which about half of the transplant patients were initially naive. However, no single risk factor can explain a majority of BKPyV reactivation events; therefore there may be as yet unidentified risk factors or a combination of risk factors that contribute to the development of PVAN.

Viral reactivation in PVAN is characterized by intranuclear viral inclusions in epithelial cells of the renal tubules, collecting ducts, and glomeruli (Nickeleit et al., 1999). The first signs of PVAN disease are increased serum creatinine levels followed by BKPyV viruria, which can be detected in 35-57% of kidney transplant recipients (Hirsch et al., 2002). BKPyV viruria can be diagnosed by PCR for viral DNA or identification of decoy cells in the urine. After viruria, 13-22% of PVAN patients develop a BKPyV viremia (Hirsch et al., 2002). The final stage of PVAN is tubular atrophy, interstitial fibrosis, and inflammation leading to potential graft loss (Hirsch et al., 2005). The gold standard for PVAN diagnosis is a biopsy of the affected kidney. However, lesions caused by BKPyV lytic reactivation can occur sporadically throughout the graft; therefore a negative biopsy result cannot rule out a PVAN diagnosis.

The current treatment for PVAN is reduction of immunosuppression, which risks graft rejection. The FDA has not approved drugs specifically for the treatment of BKPyV infection. However, several drugs that are approved to treat infections with other DNA viruses such as herpes simplex virus (HSV), CMV, and hepatitis B virus have been tested for their efficacy against BKPyV (Dropulic et al., 2010). Cidofovir, CMX001, leflunomide, fluoroquinolones, mammalian target of rapamycin (mTOR) inhibitors, and intravenous immunoglobulin (IVIG) have been used to treat BKPyV-associated diseases. Most of these drugs act by inhibiting DNA polymerases, effectively halting viral replication. Many of these drugs have been used in combination with immunosuppression reduction in patients with BKPyV reactivation. It will be important to conduct randomized clinical trials to determine which, if any, antivirals should be used in treating BKPyV-associated diseases.

Although rare, BKPyV is reported to cause other diseases in severely immunocompromised patients, such as fatal pneumonia, native kidney nephritis and encephalitis

(Cubukcu-Dimopulo et al., 2000; Galan et al., 2005; Sandler et al., 1997). BKPyV is also rarely able to cause disease in the native kidney of transplant and non-transplant patients (Nickeleit et al., 2006). More recently, there have been increased reports of native kidney BKPyV nephropathy in pediatric heart and bone marrow transplant patients (Ali et al., 2010; Sahney et al., 2010; Verghese et al., 2009).

The intracellular viral lifecycle

In order to establish an infection, BKPyV must be internalized into a permissive host cell type. The kidneys and urinary tract are the main sites of viral reactivation and replication within the host. BKPyV uses gangliosides GD1b and GT1b to attach to the cell surface and successfully infect a natural host cell, renal proximal tubule epithelial (RPTE) cells (Low et al., 2004). After binding to the cells, BKPyV is internalized via a caveolae-mediated endocytic pathway (Dugan et al., 2006). The virus then traffics to the nucleus through the ER, which contains chaperones, disulfide isomerases, and reductases that may facilitate the capsid uncoating process.

Once the uncoated viral genome enters the host cell nucleus, BKPyV viral genes are expressed in a temporal manner. All that is currently known of BKPyV transcription and replication is based on data using rearranged variants. The early and late coding regions are divergently transcribed from their promoters located within the NCCR (see Figure 4.1 in Chapter IV), which also contains the origin of replication and transcription factor binding sites (Cubitt, 2006). Canonically, the regulatory proteins, large tumor (T) antigen (TAg), small T antigen (tAg), and truncated T antigen (truncTAg) are expressed first as one primary transcript from the viral early promoter that is then alternatively spliced. TAg then binds to the origin of replication

and drives viral DNA replication, followed by expression of the late proteins VP1, VP2, VP3, and agnoprotein (Imperiale et al., 2007a). However, we and others have shown that polyomavirus late gene expression can occur prior to viral DNA replication, which will be discussed further in Chapter V (Hyde-DeRuyscher et al., 1988; Keller et al., 1984). Hyde-DeRuyscher et al. showed that even in the absence of viral DNA replication, the late promoter is always firing at a low level. Additionally, Keller et al. showed that there is low but detectable late promoter activity in the absence of both TAg and viral replication.

The TAg protein is a multifunctional regulatory protein that interacts directly with the viral genome to initiate DNA unwinding and transcription. Additionally, TAg promotes an environment of DNA synthesis in the host cell by binding to the retinoblastoma (Rb) protein and family members p107 and p130 to enhance E2F-mediated transcription of genes required to drive the cell into S phase (Felsani et al., 2006; Harris et al., 1996). Moreover, TAg binds to p53 to inhibit apoptosis and promote viral progeny production. The N-termini of TAg and tAg are shared, and therefore tAg shares some protein interactions and functions of TAg (Harris et al., 1998). However, tAg also has unique functions that control cell cycle progression such as the inactivation of protein phosphatase 2A which leads to increased levels of cyclins D1 and A, and downregulation of p27. (Pipas, 1992; Skoczylas et al., 2004; Yang et al., 1991). The truncTAg protein is a recently discovered protein whose first 133 amino acids are identical to TAg with three unique amino acids at the C terminus (Abend et al., 2009). No unique function of truncTAg has yet been defined, so it likely serves some redundant functions to TAg.

Viral DNA replication occurs following the synthesis of the regulatory proteins that promote a cellular environment conducive to replication. TAg acts as a helicase and binds to the origin of replication via multiple binding sites as a single hexamer (Tognon et al., 2003;

Yardimci et al., 2012). The viral chromatin is then unwound, the cellular DNA polymerase α /primase complex is recruited, and synthesis then proceeds from the origin in both directions. The minimal molecular requirements for SV40 DNA replication include TAg, replication protein A, DNA polymerase α /primase complex, topoisomerase I, replication factor C, DNA polymerase delta, and proliferating cell nuclear antigen (Fanning et al., 2009). These are also likely the components required for BKPyV DNA replication as well.

Robust late transcription of the structural proteins follows viral DNA replication (Cubitt, 2006). The capsid proteins VP1, VP2, and VP3 are imported into the nucleus via their nuclear localization signals. Progeny virus particles form when capsomeres assemble around newly synthesized genomes, which often form a crystalline lattice arrangement. It is not known how the virus exits the cell; however, it has been proposed that viral progeny continue to accumulate within the nucleus until the cell lyses (Drachenberg et al., 2003).

Archetype BKPyV

Currently, little is known about the replication and transcription of archetype virus, since we only recently developed a model to propagate this virus in 293TT cells (Broekema et al., 2012). As discussed in Chapter III, 293TT cells are a human embryonic kidney cell line that greatly overexpress SV40 TAg and are able to rescue replication of archetype BKPyV, which produces undetectable levels of its own TAg (Broekema et al., 2010). In contrast, rearranged variants produce abundant levels of TAg and grow readily in culture. Therefore, these variants have traditionally been used to study polyomaviruses in culture. Archetype BKPyV genomic DNA is characterized by a linear O-P-Q-R-S block NCCR structure. The O block is named for the origin of replication and the P-Q-R-S blocks are termed the enhancer region. This region

contains transcription factor binding sites for the early and late promoters. The early promoter has a defined TATA box located within the O block; however, the late promoter has no clearly defined features. This will be discussed further in Chapter IV. Rearranged variants are characterized by duplications and deletions of the sequence blocks within the NCCR.

In Chapter II, we show that the NCCR is the major genetic region that determines the replication ability of rearranged variant, and not archetype virus, in RPTE cells (Broekema et al., 2010). It is currently unclear how patterns of rearrangement contribute to replication ability. Since archetype virus is the predominant form shed in host urine, it must be capable of replication in the host. Therefore, it is possible that the permissive cell type for archetype BKPyV replication has not yet been discovered. I discuss our preliminary results of archetype virus persistence in detail in Chapter V.

Host immune responses to BKPyV

Intrinsic immunity refers to cell-based responses, usually expressed at a constitutive level, which have antiviral properties. One example of intrinsic immunity is promyelocytic leukemia nuclear bodies (PML-NBs), which are nuclear substructures possessing antiviral effects against both DNA and RNA viruses. BKPyV is thought to interfere with these antiviral effects by reorganizing PML-NBs and causing the dispersion of two of the major protein components constitutively present in PML-NBs, Daxx and SP100 (Jiang et al., 2011). Another intrinsic antiviral defense is the DNA damage response (DDR) pathway, which senses damage and coordinates repair of host genomic DNA. BKPyV activates DDR pathways, which are necessary to protect the infected cell from damage induced by the virus (Jiang et al., 2012).

The innate immune system is also important for host antiviral defense. However, currently there is a dearth of data about the interaction between BKPyV and the innate immune system. One study identified a group of pre-transplant patients with reduced dendritic cell numbers who were at increased risk of developing BKPyV viremia following kidney transplantation (Womer et al., 2010). Dendritic cells are important for antigen presentation and activation of T cells. Reduced numbers of dendritic cells could therefore impair the immune response to BKPyV. These data suggest that PVAN development may be linked to a host characteristic that predisposes an individual to BKPyV disease and highlight the importance of the innate immune system in controlling viral reactivation.

Other innate immune mechanisms, such as human antimicrobial defensins and antiviral cytokines, are important for controlling BKPyV. Defensins are small excreted peptides that form intramolecular disulfide bonds and inhibit both enveloped and nonenveloped virus infection. Human alpha defensin 5 inhibits infection of BKPyV by interacting directly with the virus particle, causing aggregation and reduced cellular attachment (Dugan et al., 2008). In addition to defensins, cytokines regulate BKPyV infection. Interferon- γ inhibits BKPyV replication by limiting viral transcription through an as yet unknown mechanism (Abend et al., 2007). However, microarray analyses of infected RPTE cells have shown that BKPyV does not induce the expression of genes of the host innate immune response such as cytokines (Abend et al., 2010). This suggests that the virus may have a means to evade or interfere with the innate response. Nevertheless, these host immune mechanisms may be compromised in a transplant patient receiving high levels of immunosuppression.

Recently, a possible active mechanism of BKPyV immune evasion was described. BKPyV and JCPyV encode a miRNA on the late strand, processed into a 5 prime (5P) and a 3 prime (3P)

arm mature miRNA, both of which target early mRNAs for degradation (Seo et al., 2008). These 3P miRNAs of BKPyV and JCPyV are identical and also target the stress-induced ligand ULBP3. ULBP3 is recognized by NKG2D, a receptor for natural killer (NK) cells. Therefore, the viral miRNA may aid in the evasion of immune recognition and killing of infected cells by NK cells (Bauman et al., 2011). In Chapter IV, we describe another role for the BKPyV miRNA in limiting replication of archetype virus. Viral miRNAs will be described in greater detail in the following section.

In addition to innate immune responses, the adaptive immune system, specifically T cell responses, are important in the control of BKPyV replication. Studies have shown that weak T cells responses are linked to recurrent BKPyV viremia, and when BKPyV-specific T cells are reconstituted, viral replication is repressed (Babel et al., 2011). However, there are also data to suggest that high levels of BKPyV-specific T cells can contribute to immunopathogenic disease in PVAN patients (Babel et al., 2011). In this study, two patients who lost their grafts due to PVAN had an accumulation of BKPyV-specific CD8 T cells in the graft. Babel et al. suggest that T cell responses may serve a beneficial role early in BKPyV infection but an immunopathogenic role late in infection (Babel et al., 2011).

Although data exist for the role of protective neutralizing antibodies as discussed earlier, humoral immunity is not thought to play a major protective role in patients with BKPyV disease. One study found that in PVAN patients, a strong cytotoxic T lymphocyte (CTL) response and low BKPyV antibody titers were associated with reduced viral loads in the blood and urine, while a weak CTL response and high BKPyV antibody titers correlated with viral load persistence (Chen et al., 2006). This suggests that the T cell response, and not the antibody response, is more important for controlling BKPyV reactivation.

The polyomavirus miRNA

As noted above, a miRNA has been discovered recently in the genome of many polyomaviruses that is encoded by the late strand and is complementary to the early coding region. miRNAs are small non-coding RNAs, approximately 22 nucleotides, that regulate gene expression post-transcriptionally by repressing translation or directing cleavage of target mRNAs (Bartel, 2004). The canonical miRNA processing pathway is initiated when the primary miRNA is transcribed in the nucleus by RNA polymerase II or III (Winter et al., 2009). The primary miRNA is then cleaved by Drosha–DGCR8 complex (Pasha), which results in a precursor hairpin RNA, termed the pre-miRNA. The pre-miRNA is exported from the nucleus to the cytoplasm, where it is cleaved to its mature length by the RNase Dicer in complex with the double-stranded RNA-binding protein TRBP. Traditionally, one strand of the mature miRNA, the functional strand, is loaded with Argonaute (Ago2) proteins into the RNA-induced silencing complex (RISC), where it guides RISC to target mRNAs. These mRNAs are then silenced through mRNA cleavage, translational repression or deadenylation.

Diverse cellular processes are regulated by miRNAs, which are generally considered fine tuners of gene expression (Baek et al., 2008). Individual miRNAs may have hundreds of targets, since they usually recognize a target with imperfect complementarity in animal cells. However, miRNAs in plants and some viruses may recognize a target with perfect complementarity. This results in direct cleavage of the target in a manner similar to small interfering RNAs (siRNAs). Although the function of many virally-encoded miRNAs remains poorly understood, their general functions can be grouped into the following categories: (1) inhibiting cell death to prolong the life of the infected cell, (2) evasion of the host immune response, and (3) promoting viral latency [reviewed in (Kincaid et al., 2012b)].

Many polyomaviruses including SV40, murine polyomavirus (MPyV), JCPyV, BKPyV, and MCPyV encode a pre-miRNA that accumulates during viral infection and targets early coding region products, such as large TAg, which drives viral DNA replication (Fanning et al., 2009; Seo et al., 2009; Seo et al., 2008; Sullivan et al., 2005; Sullivan et al., 2009). The first polyomavirus miRNA was described in SV40 and was shown to downregulate early viral gene expression (Sullivan et al., 2005). *In vitro* assays showed that cells infected with the SV40 mutant lacking the pre-miRNA are more sensitive to TAg-specific cytotoxic T cell (CTL) lysis, which suggests that the miRNA is important in evasion of the host immune response. Surprisingly, although TAg expression was increased in this mutant, replication was unaffected. A naturally occurring miRNA deletion mutant in MPyV has no defects with respect to replication, transformation efficiency, and establishment or clearance of infection *in vivo* (Sullivan et al., 2009). As a result, the MPyV miRNA was deemed non-essential for *in vitro* and *in vivo* infection. The MPyV miRNA did not limit the host CTL response during either the acute or persistent phases of *in vivo* viral infection. Therefore, there is no clearly defined role conserved among all polyomavirus miRNAs.

JCPyV and BKPyV encode one pre-miRNA on the late strand that has perfect complementarity to the 3' coding end of the TAg mRNA (Seo et al., 2008). Since the miRNA is located on the late strand, it may be expressed from the late promoter, although it is also possible that it has a unique promoter located within or outside the NCCR. The location of the elements that regulate miRNA expression will be discussed in more detail in Chapter IV. In animals, one arm of the miRNA duplex is degraded while the other arm is preferentially loaded into RISC (Bartel, 2004). However, both arms of the BKPyV and JCPyV miRNA direct degradation of the TAg mRNA target in an siRNA-like fashion (Seo et al., 2008). As discussed earlier, the JCPyV

and BKPyV 3P miRNAs have also been implicated in host immune evasion by targeting the ligand ULBP3 to escape NKG2D-mediated recognition and killing of infected host cells (Bauman et al., 2011). Prior to the work described in this dissertation, no function, resulting from miRNA-induced TAg downregulation had been assigned to the JCPyV or BKPyV miRNAs.

Summary and chapter outline

BKPyV is ubiquitous in the human population, where it establishes a persistent subclinical infection in the urinary tract. Under conditions of immunosuppression, the virus can reactivate to cause disease and its NCCR structure may rearrange by an as yet undefined mechanism to promote virus replication (Gosert et al., 2008a). Previously, our lab developed a cell culture system to study lytic replication of the BKPyV rearranged variant, in primary RPTE cells (Low et al., 2004). While rearranged variants replicate robustly in this natural host cell, archetype virus is unable to do so. Therefore the primary goal of this thesis research is to understand the mechanisms regulating archetype polyomavirus replication, the transmissible form that establishes persistence in the human host. We hypothesize that the replication of archetype BKPyV, unlike rearranged variants, is uniquely restricted by the action of a regulatory miRNA whose expression is controlled through differential promoter activity. We believe that this may play a key role in the establishment or maintenance of viral persistence.

The BKPyV genome is divided into three major regions: the early coding region, the late coding region, and the NCCR. In Chapter II we demonstrate that the NCCR determines the replication phenotype in RPTE cells, differentiating rearranged variants and archetype virus. To examine the contribution of each genetic region to replication ability, a swap vector system was created to test all possible combinations of regions between archetype virus and rearranged

variant. We show that the NCCR alone confers replication ability of the rearranged variant in an archetype virus background and that the archetype virus NCCR is sufficient to confer loss of replication in the rearranged variant background. Additionally, archetype virus produces no detectable TAg in RPTE cells, which is required to initiate viral replication. We also tested the NCCRs from clinical isolates and showed that replication increased in correlation to the rearrangement complexity.

In Chapter III we describe the use of 293TT cells, which express high levels of SV40 TAg, as an artificial cell culture model to propagate archetype polyomaviruses. Using this model to propagate archetype BKPyV, we then investigated the role of the miRNA in limiting archetype virus replication early during infection in RPTE cells and present preliminary data on the location of the BKPyV miRNA regulatory elements as discussed in Chapter IV. Chapter V includes a summary of the results, future areas of study, and conclusions including a proposed model overview. Finally, the appendix describes a potential model of archetype BKPyV persistence in urothelial cells.

Notes

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Chapter II

A system for the analysis of BKPyV non-coding control regions: application to clinical isolates from an HIV/AIDS patient

Abstract

BK polyomavirus (BKPyV) is an important opportunistic human pathogen whose disease prevalence continues to increase with the growing immunocompromised population. To date, the major determinant of replication in cell culture has not been formally proven. BKPyV exists as archetype virus and rearranged variants, which are classified based on the DNA sequence of their non-coding control regions (NCCRs). The archetype BKPyV NCCR is divided into five blocks of sequence and rearranged variants contain deletions and duplications of these blocks. In this study, a genetic system was developed and used to identify the major determinant of replication ability in primary renal proximal tubule epithelial cells, the natural host cell of BKPyV. This system was also used to analyze NCCR variants isolated from an immunocompromised patient which contain assorted rearrangement patterns and functional differences. This study solidifies the NCCR as the major genetic determinant of BKPyV replication ability *in vitro*.

Introduction

BK polyomavirus (BKPyV) is a highly prevalent human polyomavirus originally isolated from the urine of a renal transplant patient with ureteric obstruction (Gardner et al., 1971).

BKPyV is believed to be spread asymptotically by a respiratory route, based on evidence that seroconversion takes place in early childhood and that BKPyV has been detected in tonsillar and salivary gland tissues (Goudsmit et al., 1982; Jeffers et al., 2009). However, data also suggest that a fecal-oral route of transmission may be possible (Vanchiere et al., 2009; Vanchiere et al., 2005a; Wong et al., 2009). The seroprevalence of BKPyV in the population is reached by the age of ten and is estimated to be between 65-90% (Kean et al., 2009; Knowles, 2006). Following primary infection, BKPyV establishes a lifelong, persistent, subclinical infection in the urinary tract (Chesters et al., 1983; Heritage et al., 1981a). Renal tubule epithelial cells are a major site in which BKPyV persists and can be reactivated (Knowles, 2001; Moens et al., 2001). BKPyV is not known to cause disease in healthy, immunocompetent people, but it can be periodically isolated from their urine as a result of transient reactivation events (Knowles, 2001). In some clinical settings, BKPyV can reactivate and cause diseases such as hemorrhagic cystitis (HC) in bone marrow transplant patients and polyomavirus-associated nephropathy (PVAN) in kidney transplant patients. BKPyV can also reactivate in other immunosuppressed populations such as pregnant women and patients with HIV/AIDS (Doerries, 2006). Studies have shown both increased incidence and amount of virus present in the urine of HIV-infected patients as compared to uninfected people (Knowles et al., 1999; Markowitz et al., 1993). Although rare, BKPyV infection in HIV/AIDS patients can result in death (Cubukcu-Dimopulo et al., 2000).

BKPyV has a small double-stranded circular DNA genome that is divided into three genetic regions: the early coding region, the late coding region, and the non-coding control region (NCCR). BKPyV is classified into archetype virus and rearranged variants, based on the structure of the NCCR, which contains the origin of DNA replication and promoters for both the early and late coding regions. Archetype BKPyV is characterized by five blocks of DNA

sequence within the NCCR; O, P, Q, R, and S (Figure 2.1A). The O block contains the origin of replication and putative transcription factor binding sites. Rearranged variants of BKPyV are characterized by deletions and duplications in the sequence blocks that enhance replication ability of the virus and allow it to grow readily in cell culture. The early and late gene promoters and transcriptional enhancers, as defined in rearranged variants, are located in the P, Q, R, and S blocks (Markowitz et al., 1988; Moens et al., 1995; Moens et al., 2005). BKPyV archetype virus can be isolated from both healthy individuals and diseased patients, and it currently cannot be grown in cell culture. Rearranged BKPyV variants are most commonly isolated from patients with BKPyV diseases such as PVAN and HC, but not in all such patients, and replicate robustly in culture.

Previous studies have shown that when archetype virus, isolated directly from patient urine, is passaged for prolonged periods in cell culture, it accumulates rearrangements in the NCCR (Rubinstein et al., 1991). Likewise, although the archetype virus is the prevalent form of BKPyV, when high titers of virus are present in the serum of a patient with BKPyV disease, the appearance of rearranged variants increases and these eventually replace archetype as the predominant form of the virus (Gosert et al., 2008b). This is likely due to increased replication efficiency of rearranged variants, resulting in the ability to outcompete archetype virus in an immunocompromised host. The dominance of rearranged variants in a host may directly relate to clinical outcome, since these variants are able to replicate more efficiently and show increased cytopathology *in vitro* (Gosert et al., 2008b). A recent study showed that multiple rearranged quasispecies can be found in the urine or allograft biopsies of kidney transplant recipients, within a single patient (Olsen et al., 2009). In addition to differences in the NCCRs between archetype virus and rearranged variants, it is possible that polymorphisms in the early and late coding

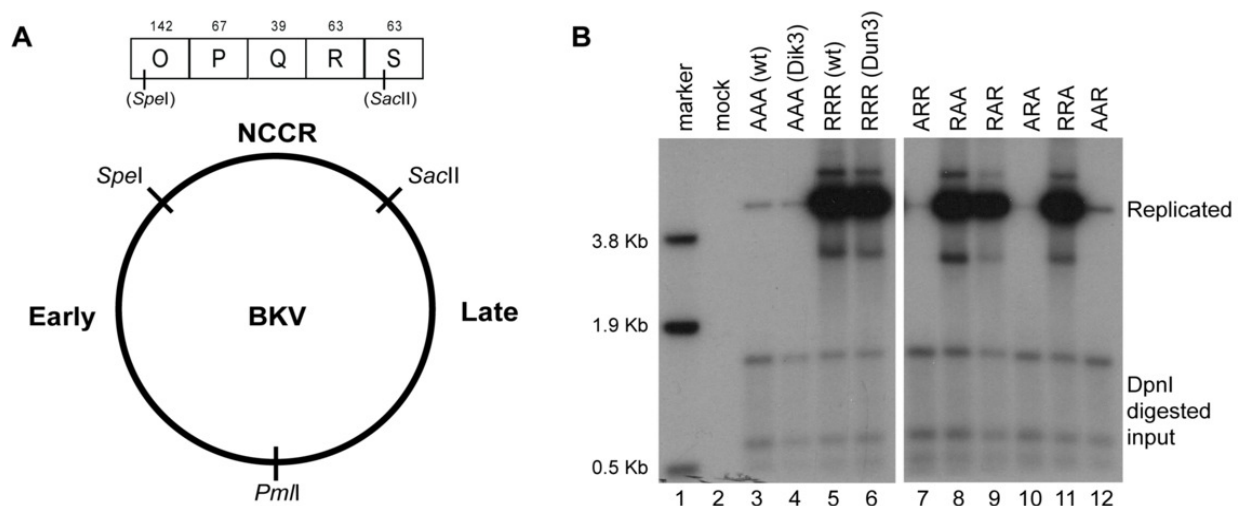


Figure 2.1. The NCCR determines replication efficiency of BKPyV. (A) Schematic of the swap genome with the archetype virus (Dik) NCCR. SpeI and SacII sites were inserted into the pBR322-Dunlop or -Dik vectors flanking the majority of the NCCR and a PmlI site was inserted between the early and late regions. (B) RPTE cells were transfected with recircularized viral genome and low molecular weight DNA was harvested 5 dpt. Samples were linearized, digested with DpnI, and analyzed by Southern blotting. The left panel shows Dik and Dunlop wt replication compared to the swap vectors containing the three inserted restriction enzyme sites, Dik3 and Dun3 respectively. The right panel shows all possible swap combinations. Each construct is designated by a three letter abbreviation. A=archetype and R=rearranged. The first letter denotes the NCCR; second letter, early region; third letter, late region. Marker, HindIII digest of pGEM-TU; Mock, mock transfection.

regions can also contribute to replication ability. Comparing the Dik archetype virus (Goudsmit et al., 1981) and the Dunlop rearranged variant (Seif et al., 1979), for example, there are four amino acid differences in the early coding region: three in TAg and one in tAg. There also are nine amino acid differences in the late coding region: three in VP1 and six in VP2, three of which overlap in VP3. However, contributions of the NCCR, the early coding region, and late coding regions to replication have not been systematically examined.

For the present study, a swap vector system was developed to resolve the major determinants of BKPyV replication in primary renal proximal tubule epithelial (RPTE) cells. This system allows for independent analysis of the major viral DNA functional regions, the NCCR and the early and late coding regions, from different BKPyV isolates. Using this system, we were able to formally prove that the NCCR is the major determinant of replication in cell culture. The swap vector system was then used to functionally analyze variants isolated from the urine of an AIDS patient with BKPyV viremia. These clones displayed a series of NCCR rearrangements with variable levels of replication ability and infectious progeny production. These studies provide a useful genetic system with which to analyze patient isolates and dissect specific determinants of BKPyV replication.

Results

The NCCR is sufficient to determine replication ability in cell culture. BKPyV variants with rearranged NCCRs are able to replicate in cell culture while viruses with an archetype NCCR are unable to do so. To determine whether other regions of the BKPyV genome contribute to replication ability, we created a swap vector system that allows for the exchange of the early coding region, late coding region and the NCCR between archetype virus and rearranged variants

(Figure 2.1A). Unique restriction sites were introduced into the archetype (Dik) and rearranged (Dunlop) variant genomes to create the swap vectors and investigate the contributions of the three regions to the replication ability in RPTE cells. SpeI and SacII sites were introduced into regions with no sequence variation between Dik and Dunlop in the O block and the S block, respectively, flanking the majority of the NCCR. Additionally, a PmlI site was inserted between the early and late regions in both the archetype and rearranged genome in a pBR322 backbone (Seif et al., 1979). The swap vectors were named Dik-3-site (Dik3), and Dunlop-3-site (Dun3), in reference to the insertion of the three restriction sites. Using these vectors, we constructed all possible combinations of the three regions of the archetype virus and rearranged variants. We were then able to assay replication ability by a DpnI resistance assay, which distinguishes methylated transfected plasmid DNA that has not been replicated in eukaryotic cells from unmethylated DNA that has been replicated in eukaryotic cells (Pipas et al., 1983).

Each recombinant genome was excised from the plasmid, re-circularized, and transfected into RPTE cells. At five days post-transfection, low molecular weight DNA was isolated (Hirt, 1967), linearized, digested with DpnI, and assayed by Southern blotting (Figure 2.1B). The exogenous restriction sites had no impact on the replication ability of the genome (compare lanes 3 to 4 and lanes 5 to 6). Only those recombinants created containing the rearranged variant NCCR were able to replicate efficiently (lanes 8, 9, and 11) as compared to recombinants containing the archetype NCCR (lanes 7, 10, and 12). Of note, a low level of archetype replication was seen in lanes 3, 4, 7, 10, and 12; however no infectious progeny were detected by an infectious unit assay (Figure 2.3C). What appears to be low level archetype virus replication could have been the result of incomplete DpnI digestion of input DNA. These data demonstrate that the NCCR is the major determinant of the ability to replicate in RPTE cells.

Characterization of BKPyV NCCR variants isolated from an immunocompromised patient. To test the utility of this system for analyzing patient isolates, we cloned the BKPyV NCCR from a urine sample of an AIDS patient with BKPyV viremia. The serum and the urine of this patient were tested for the presence of BKPyV and JC polyomavirus (JCPyV) by quantitative PCR as previously described (McNees et al., 2005). JCPyV was not detected in either fluid and BKPyV was only detected in the urine, at 150 genomes/mL. The BKPyV amplicons from the patient were sequenced and classified as genotype Ia based on VP1 polymorphisms (Jin et al., 1993b; Zheng et al., 2007). We were able to isolate five distinct NCCRs from this single urine specimen displaying differing degrees of rearrangement (Figure 2.2A). These clones were designated TCH for their location of isolation (Texas Children's Hospital) and numbered 1-5. Clones TCH3 and TCH4 have a partial P block inserted before a complete P block, and differ from each other by the presence of a point mutation. Clones TCH1 and TCH2 each have unique truncations in their R blocks. In addition, TCH1 is completely missing the S block. The most highly rearranged clone, TCH5, contains a partial duplication of both the P and the Q blocks inserted between the full P and Q blocks. All five also have a single nucleotide polymorphism in the O block as compared to both Dik and Dunlop.

We first assessed the replication ability of each isolate by introducing the restriction enzymes SpeI and SacII into the NCCR of each variant and then swapping them into the Dun3 backbone. All genomes were excised from the plasmid backbone, re-circularized, and transfected into RPTE cells. Four days post-transfection, low molecular weight DNA was isolated and assayed for replication ability by Southern blotting (Figure 2.3A). Day 4 was chosen because

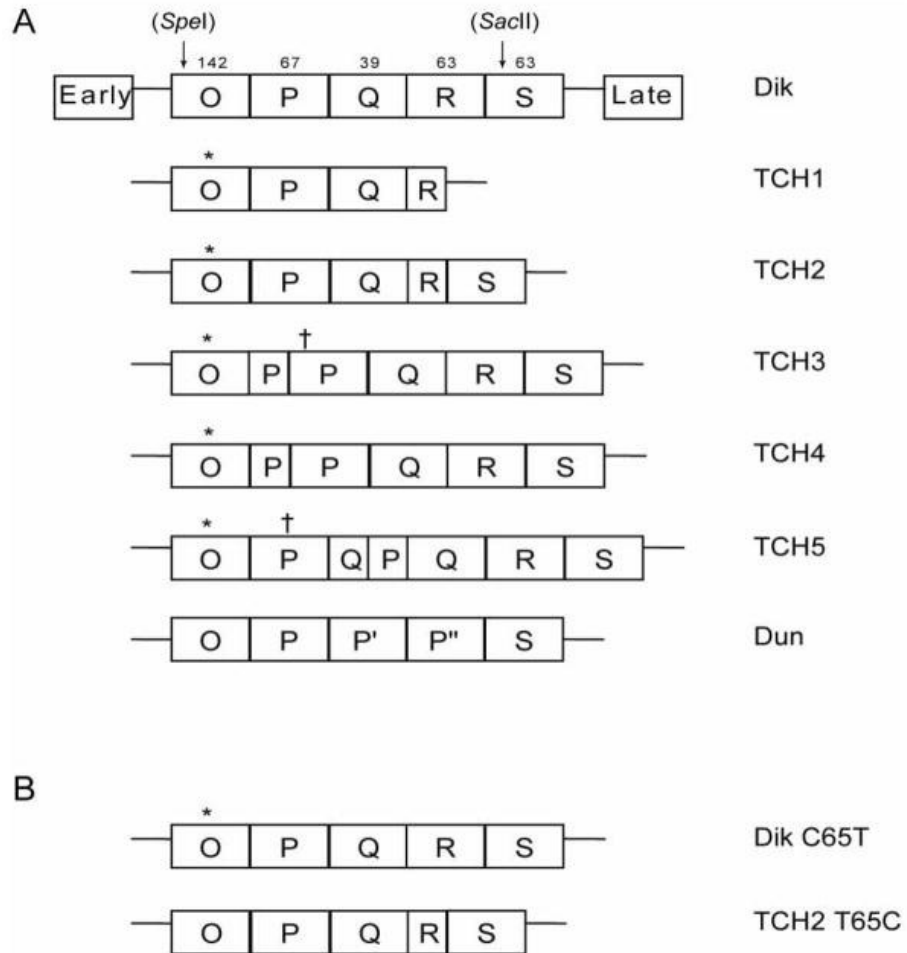


Figure 2.2. Schematic of NCCRs. (A) Schematic of the NCCRs of viruses isolated from a single clinical specimen. The NCCR is divided into blocks of sequence O, P, Q, R, and S. The block structures of the archetype virus (Dik), the rearranged variant (Dun), and the variants isolated from the patient are shown. The * represents the O block polymorphism and the † represents other mutations relative to the archetype P block sequence. The P' and P'' blocks of Dunlop are variations of the P block. (B) A schematic of the Dik C65T and TCH2 T65C mutants created by site directed mutagenesis.

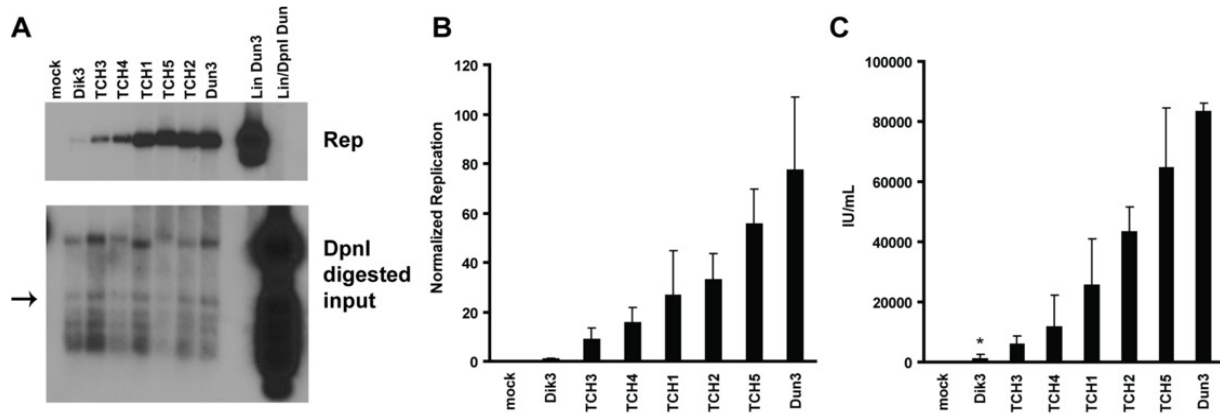


Figure 2.3. Clones isolated from a clinical specimen display variable replication abilities. RPTE cells were transfected with recircularized viral genomes. (A, B) Low molecular weight DNA was harvested 4 dpt and analyzed as in Fig. 1. (A). Representative Southern blot. The two panels are different exposures of the same blot. Arrow indicates the DpnI-digested band used for normalization among samples. Mock, mock transfection. (B) Quantification of replication data from three independent experiments, error bars represent standard error of the mean. (C) Viral lysates were prepared from parallel transfections at 7 dpt and assayed for infectious progeny as described in the Materials and Methods. Results are from three independent experiments, error bars represent standard error of the mean. The * represents a measurement below the effective limit of detection.

parental rearranged variant viral replication can be easily detected at this time point (Figure 2.3A). To quantify replication ability, the replicated genome band was normalized to a DpnI-digested input band (Figure 2.3A, arrow) to control for transfection efficiency and the archetype virus (Dik3) was set to one (Figure 2.3B). The clones show various genome replication abilities ranging from low and intermediate (TCH1, TCH3, TCH4, TCH2) to high (TCH5), which replicated to levels similar to Dun3.

To further characterize these patient isolates, infectious progeny production was assessed using an infectious unit assay as previously described (Low et al., 2004). Each recombinant genome was transfected as described above and cell lysates were harvested seven days post-transfection. Progeny production followed the same trend as replication, with low and intermediate level replicating clones producing less progeny than the high level replicating clone (Figure 2.3C). These data indicate that NCCR rearrangements are able to account for functional differences assayed *in vitro*.

Finally, we determined whether the O block polymorphism (C65T where 1 is the O block starting position) affected replication. Site-directed mutagenesis was used to recreate the polymorphism common to all the patient variants in an archetype virus background (Figure 2.2B). Additionally this polymorphism was reverted to the Dik sequence in a TCH2 background. Replication ability was assessed by DpnI resistance assay as above. Results from this assay show that the point mutation does not affect the replication ability of the viruses (Figure 2.4).

Discussion

The determinants that regulate the ability of archetype virus and rearranged variants of BKPyV to replicate in cell culture have not yet been systematically mapped. In human embryonic kidney

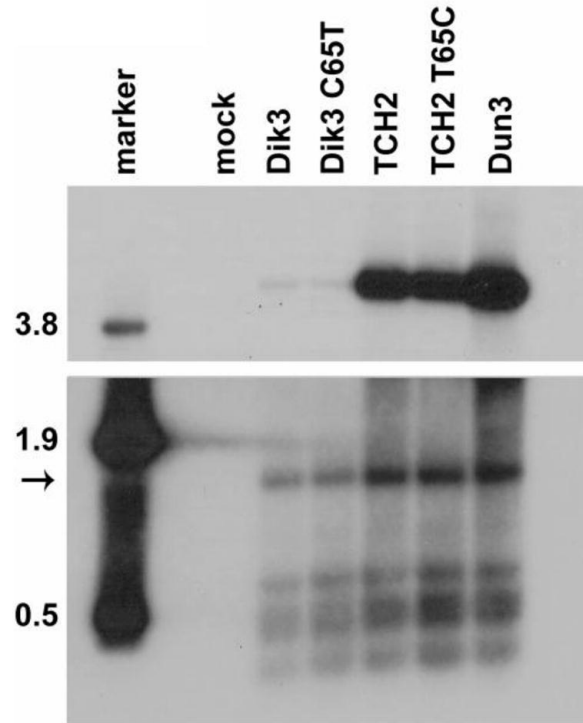


Figure 2.4. The O block polymorphism does not affect replication ability. The C65T mutation was created and reversed in the O blocks of Dik3 and TCH2, respectively, and the genomes were assayed as in Fig. 3A. The blot is representative of two independent repeats, and the two panels are different exposures of the same blot. Marker: HindIII digest of pGEM7-TU (size of bands in kb); Mock, mock transfection.

and adult human skin fibroblast cells, which are not natural target cells for BKPyV, prolonged passage of archetype virus results in the accumulation of NCCR rearrangements leading to increased replication ability (Rubinstein et al., 1991). Thus, the NCCR was assumed to be the main determinant of viral replication based on the data that rearrangements are apparently selected for in cell culture; however, this research was performed in cells that are not the natural host of BKPyV infection. Furthermore the dependence of replication ability on the NCCR has not previously been directly proven. Additionally, there is suggestive evidence for the contribution of the early and late coding regions in regulating polyomavirus replication ability. For example, a naturally occurring Q169L mutation in the BKPyV large tumor antigen (TAg) protein has been proposed to stabilize its binding to DNA, which could conceivably affect replication ability since TAg stimulates viral replication (Smith et al., 1998). Of note, this mutation is not present in either the Dik or Dunlop viruses used in the present study.

In this study, we showed directly that the NCCR is the major determinant of BKPyV replication in cell culture. To answer the question of which region determines viral replication ability, we developed a swap vector system. This allowed us to create recombinants of the archetype virus and rearranged variant genomes to assess the roles of the NCCR, early coding region, and late coding region in replication. Insertion of restriction enzyme sites between the regions of the genome did not affect replication. We then used this vector system and demonstrated that the NCCR is the major determinant of viral replication ability in RPTE cells because only those recombinants containing a rearranged variant NCCR were able to replicate efficiently. This result seems logical considering that the NCCR, early and late coding regions all have functions that are required for viral replication, but the early and late coding regions are more genetically constrained. For example, genetic instability in TAg would be problematic

because it needs to be able to bind the origin in the NCCR to initiate replication (Eichman et al., 2004). TAg also has many other functions that require interaction with host proteins, suggesting that the TAg sequence needs to be stable to maintain these interactions. The same restrictions may be seen for the late coding region since the capsid is a relatively rigid structure whose main component is the VP1 protein, which needs to be able to recognize host gangliosides for entry into the cell (Low et al., 2006).

We used the swap vector system to analyze BKPyV NCCRs cloned directly from a single urine specimen from an AIDS patient with BKPyV viraemia. The NCCRs of five distinct isolates with various rearrangements were introduced into the Dunlop background in order to analyze replication ability and infectious progeny production. All five clones were able to replicate better than the archetype virus, Dik, and each contained at least partial duplications or deletions in the NCCR sequence blocks. In general, the isolates that had a greater degree of rearrangements had higher replication ability and infectious progeny production than those with fewer rearrangements. However, with such a small sample size it was difficult to discern a clear pattern of whole or partial block rearrangements that correlated to replication ability. Clones TCH3 and TCH4 differed by only a point mutation in the P block and displayed similar functional profiles. There was no archetype virus isolated from this patient's urine but this does not confirm its absence: due to the limits of PCR-based assay detection, only the most highly represented variants are likely to be detected. The absence of archetype virus from this patient's urine sample may indicate the severity of the patient's immunocompromised state, however. The presence of rearranged BKPyV variants in serum is associated with increased replication and inflammation (Gosert et al., 2008b). While virus excreted in the urine may be produced at a different site of replication from serum, it may still follow the same trend as the virus found in the serum with

respect to clinical severity. Additionally, the HIV-1 Tat protein is able to activate the BKPyV early promoter in *trans* through sites located in the NCCR (Gorrill et al., 2006). This activation increases TAg expression and therefore, viral DNA replication. This is another possible factor influencing the reactivation of BKPyV in HIV-infected patients.

Further study is needed to determine why rearranged variants, and not archetype virus, are able to replicate in cell culture. Archetype BKPyV is more frequently isolated from patients than rearranged variants, but there are no reports of successfully culturing this form. It is possible that archetype virus and rearranged variants naturally replicate in separate populations of cells in the host, such that proximal tubule cells are permissive to rearranged variants but not archetype virus. Another possible, but not mutually exclusive, explanation is that the presence or absence of certain transcription factor binding sites in the NCCR of rearranged variants is required for replication in tissue culture. We were unable to identify any obvious pattern contributing to replication ability from an analysis of the variant NCCRs in comparison to the archetype virus using a transcription factor binding site prediction program, AliBaba2.1 (see <http://www.gene-regulation.com/pub/programs.html>).

In addition to NCCR rearrangements affecting viral gene transcription, it is possible that there are direct effects on DNA replication of the virus. It has been shown that there are three TAg binding sites within the SV40 regulatory region (i.e., the SV40 NCCR) that are assumed to be present in the BKPyV NCCR as well (Del Vecchio et al., 1989; Yang et al., 1979). Rearrangements in the SV40 regulatory region affect the binding stability of TAg to these sites, altering the DNA conformation and the levels of DNA replication (Wilderman et al., 1999). Also, *cis*-acting sequence elements in mouse polyomavirus and SV40 are involved in the activation of replication (de Villiers et al., 1984; O'Connor et al., 1988; Tang et al., 1987).

Although the O block polymorphism C65T, which is located in putative TAg binding site I, is present in all of the BKPyV variants in the present study, this mutation did not significantly affect replication ability. We can use the swap vector system in the future to further characterize the role of the rearranged NCCR by dissecting its contribution to transcription and replication.

Materials and Methods

Patient and Sample Collection. Urine specimens were collected periodically from subjects enrolled in a prospective study of polyomavirus excretion in the urine of immune compromised patients. The study protocol was approved by the Institutional Review Board for Human Subjects Research at Baylor College of Medicine. Informed consent and assent, when appropriate, was obtained from subjects prior to specimen collection in 2001. The specimens in this report are from a single patient.

Cell Culture. Primary human RPTE cells were maintained for up to six passages in renal epithelial growth medium as previously described (Abend et al., 2007). Infectious unit assays were performed as previously described (Low et al., 2004).

Plasmids. The pBR322-Dunlop plasmid (ATCC #45025, pBKV) was received from Peter Howley (Seif et al., 1979). The pBR322-Dik plasmid was received from John Lednicky. Both of these genomes are inserted into the pBR322 backbone at the BamHI site. Site-directed mutagenesis was performed using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene) as described previously (Abend et al., 2008) on both pBR322-Dik and Dunlop to create Dik-3-site (Dik3) and Dunlop-3-site (Dun3) swap vectors. The SpeI site was inserted into the O block using primer A and its reverse complement and the SacII site was inserted using primer B and its reverse complement (Table 2.1). The PmlI site was inserted between the early and late regions

Table 2.1. List of primers used for site-directed mutagenesis and splicing by overlapping extension PCR^a.

	Primer	Sequence (5'-3')
A	NCCR early SpeI	GGGGAAATC <u>ACTAGT</u> CTTTT G CAATTTT G CAAAAATGG
B	NCCR late SacII	GACAAGGCCAAGATT <u>CCGCGG</u> CTCGCAAAACATGTC
C	Tag PmlI	ACACCACCCCCAAAATAACAC G TGCTTAAAAGTGGCTTATAC
D	NCCR bridge For	TAGTAAGGGTGTGGAAGCT
E	NCCR bridge Rev	CCATGGATTCTTCCCTGTTA
F	TCH1 SacII	GACAAGGCCAAGATT <u>CCGCGG</u> GAGAATTTTAGGGGCGG
G	Dik3 C65T	GGCCTCAGAAAAAGCTTCCACACCCTTACTAC
H	TCH2 T65C	GGCCTCAGAAAAAGCCTCCACACCCTTACTAC

^a Restriction enzyme sites are underlined and point mutations are shown in bold in the primer sequence.

using primer C and its reverse complement. Swap combinations were created by digesting 10 µg of Dik3 and Dun3 with the appropriate restriction enzymes to obtain the desired fragment (NCCR, early, or late). Each fragment was then gel extracted and ligated with its counterpart genome using T4 DNA ligase at a plasmid to fragment ratio of 1:2. All plasmids were confirmed by DNA sequencing.

Site-directed mutagenesis was performed on both Dik3 and the TCH2 clone to create and revert to O block polymorphism found in clinical variants, respectively. The C to T mutation was made in Dik3 using Primer G and its reverse complement and the T to C mutation was made in TCH2 using Primer H and its reverse complement (Table 2.1). Mutations were confirmed by DNA sequencing. The integrity of the early coding region was also confirmed by DNA sequencing.

BKPyV-NCCRs were amplified by PCR as previously described (Vanchiere et al., 2005c) from the urine of a patient with AIDS and BKPyV viremia. NCCR amplicons were cloned using the TOPO-TA system as directed by the manufacturer (Invitrogen, Carlsbad) and then into the Dun3 backbone. The Dun3 O block, containing the origin of replication and the SpeI site was added to each NCCR fragment by a Splicing by Overlap Extension (SOE) PCR reaction (Horton et al., 1989). Additionally a SacII site was added to the existing S block of each NCCR by site directed mutagenesis. However, the SacII site was added into the truncated R block in the TCH1 clone because the S block is deleted in this clone. A bridge piece containing the O block and SpeI site was created by amplifying that region of the Dun3 plasmid using primers D and E. The bridge piece and the NCCR fragment were combined in a melting and slow cooling reaction using the program: 5 min at 95°C; 40 cycles, 30 sec each of a gradient from 95°C to 55°C; annealing at 68°C for 5 min. The forward SacII primer B, or F for clone TCH1, and the reverse

SpeI primer A were then added to the above reaction at 4 nM each and PCR amplification was performed using the program: 3 min 30 sec at 94°C; 30 cycles of 45 sec at 94°C, 1 min at 66°C, 1 min at 68°C, annealing and extension at 68°C for 7 min. Fragments were then swapped into the Dun3 backbone as described above. GenBank accession numbers for the NCCRs are as follows: TCH1, HQ148166; TCH2, HQ148167; TCH3, HQ148168; TCH4, HQ148169; TCH5, HQ148170.

Transfections. The viral genome was excised from the pBR322 vector by BamHI (New England Biolabs, Ipswich, MA) digestion and then recircularized at a concentration of 10 ng/μL using T4 DNA ligase (New England Biolabs, Ipswich, MA). RPTE cells were seeded into 12 well plates and transfected with 0.6 μg DNA at 60-70% confluency using TransIT LT-1 transfection reagent (Mirus Bio, Madison, WI) according to the manufacturer's instructions with a DNA to transfection reagent ratio of 1:6. Transfection complexes were removed 18 h post-transfection by washing once with PBS and adding fresh media.

Southern Blotting. Low molecular weight DNA was harvested four or five days post-transfection (dpt) by the Hirt protocol (Hirt, 1967). DNA was linearized by digesting with BamHI or EcoRI and then digested with DpnI, which cuts only methylated DNA that has not been replicated in eukaryotic cells (Pipas et al., 1983). The sample was separated on a 1% agarose gel in 1X TAE buffer (40 mM Tris acetate and 1 mM EDTA, pH 7.8). The gel was washed with depurination solution (0.25 M HCl for 30 min), followed by denaturation solution (0.5 M NaOH, 1.5 M NaCl, twice for 20 min), followed by neutralization solution (0.5 M Tris-HCl, pH 7.4, 1.5 M NaCl, twice for 20 min). The gel was transferred overnight to nylon membrane (Gene Screen Plus, Perkin Elmer) in 20X SSC using capillary transfer. The membrane was washed for 5 min in 6X SSC and exposed to UV light for 2 min, then baked for 2

h at 80°C to fix the DNA to the membrane. The probe used was a 3239 bp BKPyV PvuII fragment excised from pGEM7-TU (another rearranged variant of BKPyV) which spans the early region and the NCCR, and was labeled with the Random Primers DNA Labeling System (Invitrogen). The membrane was rehydrated in 6X SSC and incubated for 1 h at 68°C in prehybridization solution (5X SSC, 1X Denhardt's solution, 1% SDS) with 0.1 mg/mL denatured sheared salmon sperm DNA. The membrane was incubated overnight at 68°C in hybridization solution (5X SSC, 1X Denhardt's solution, 1% SDS) with 0.1 mg/mL denatured sheared salmon sperm DNA and 1×10^8 counts per minute of labeled probe. After hybridization, the membrane was washed with 2X SSC with 0.1% SDS twice for 5 min at room temperature followed by 0.2X SSC with 0.1% SDS twice for 5 min at room temperature and twice for 15 min at 42°C. The membrane was then rinsed with 2X SSC before being exposed to film. The Southern blot was quantified using a Typhoon phosphoimager and Imagequant software (GE).

Notes

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Chapter III

Efficient propagation of archetype BK and JC polyomaviruses

Abstract

BKPyV and JCPyV are closely related, ubiquitous human pathogens that cause disease in immunocompromised patients. The DNA sequence of the regulatory regions distinguishes two forms of these viruses, designated archetype and rearranged. Although cell culture systems exist for rearranged BKPyV and JCPyV, currently there is no robust cell culture system to study the archetype viruses. Large T antigen (TA_g) is a virally encoded protein required to initiate viral DNA synthesis. Because archetype virus produces undetectable levels of TA_g, we hypothesized that TA_g overexpression would stimulate archetype virus replication. Efficient propagation of the archetype forms of BKPyV and JCPyV was observed in 293TT cells, human embryonic kidney cells overexpressing SV40 TA_g. Importantly, the archetypal structure of the regulatory region was maintained during viral growth. Significant replication was not observed for Merkel cell, KI, or WU polyomaviruses. 293TT cells provide a means of propagating archetype BKPyV and JCPyV for detailed study.

Introduction

BK polyomavirus (BKPyV) and JC polyomavirus (JCPyV) are closely related human viruses originally isolated by two independent groups from patient samples in 1971 (Gardner et al., 1971; Padgett et al., 1971). BKPyV, JCPyV, and the related simian virus 40 (SV40) all share

close to 70% whole genome sequence identity and differ mainly in their regulatory or transcriptional control regions (Imperiale, 2001). Both BKPyV and JCPyV are thought to be contracted in childhood or early adulthood. For BKPyV, the adult population seroprevalence is reached by the age of 10 and is estimated to be between 65% and 90% (Kean et al., 2009; Knowles, 2001). For JCPyV, the seroprevalence is lower than that of BKPyV, roughly 20% by the age of ten, and increasing to up to about 50% by the age of 70 (Kean et al., 2009). After what is thought to be a primary asymptomatic infection, these viruses disseminate to and persist in the urinary tract. In immunosuppressed patients, the viruses can reactivate and cause serious disease in specific sites. BKPyV can reactivate in bone marrow transplant patients to cause hemorrhagic cystitis in the bladder, and in kidney transplant patients to cause polyomavirus-associated nephropathy in the kidney (Ahsan et al., 2006). JCPyV can cause the fatal demyelinating disease, progressive multifocal leukoencephalopathy (PML), in which viral growth is restricted to glial cells of the brain, particularly in AIDS patients (Dorries et al., 1979).

BKPyV and JCPyV are small, non-enveloped, icosahedral viruses with circular double-stranded DNA genomes (Imperiale et al., 2007b). The genomes are divided into three genetic regions: the early coding region, the late coding region, and the regulatory region, which is also called the non-coding control region (NCCR) in BKPyV. The regulatory region contains the origin of DNA replication and the promoters for the divergently transcribed early and late coding regions. The early coding region encodes the non-structural T antigen proteins that are responsible for inducing a cellular environment conducive to replication and initiating viral DNA synthesis. The late coding region encodes the structural capsid proteins VP1, VP2, and VP3, and agnoprotein.

Two forms of the viral genomes of BKPyV and JCPyV exist, which are designated archetype and rearranged based on the DNA sequence of the regulatory region. The archetypal regulatory region is divided into defined blocks of sequence that are duplicated or deleted in the rearranged variants. In BKPyV, the archetype virus NCCR is divided into five blocks of sequence designated O, for the origin of replication, P, Q, R, and S (Moens et al., 2005). The JCPyV archetype regulatory region is similarly divided into blocks of sequence designated a, b, c, d, e, and f, with the origin of replication located between block a and the early genes (Jensen et al., 2001). The archetype form of both viruses is thought to be the transmissible form that is capable of establishing a persistent infection in the host. For BKPyV, the archetype form can be periodically isolated in the urine of healthy individuals as a result of transient reactivation events (Doerries, 2006; Knowles, 2001). In contrast, rearranged variants are most often isolated from the serum of patients with BKPyV disease (Gosert et al., 2008b). Similarly, JCPyV archetype virus isolation is often limited to the urine or urinary tract sites such as the kidney in healthy individuals (Loeber et al., 1988; Yogo et al., 1990). The rearranged form of JCPyV can be isolated from the brain and lymphocytes in addition to the kidneys and urine in people with and without PML (Loeber et al., 1988; Tornatore et al., 1992; White et al., 1992; Yogo et al., 1990). Rearranged variants of BKPyV and JCPyV are able to grow readily in the current cell culture models of renal proximal tubule epithelial (RPTE) cells and human fetal glial cells, respectively (Low et al., 2004; Padgett et al., 1977). To date, cell culture systems to study the archetype form of BKPyV and JCPyV have been extremely limited. However, it is important to be able to grow the archetype form of these polyomaviruses to understand the natural history of infection, develop means to prevent transmission and reactivation, and to aid in the search for a naturally permissive cell type for archetype virus in the host.

There is only one report of archetype BKPyV propagation in cell culture, but that propagation was not efficient. Archetype virus was shown to have limited replication capacity in a human endothelial cell line (Hanssen Rinaldo et al., 2005). Infectious progeny were not detected until 35 days post transfection (dpt) and only 4% of cells were infected upon secondary infection of the endothelial cells regardless of the multiplicity of infection of the inoculum. Additionally, the authors showed that while the archetype virus NCCR configuration was maintained, there were a significant number of rearranged variants identified in the progeny. Attempts to culture archetype JCPyV have been somewhat more successful. Archetype JCPyV has weak DNA replication ability in POJ cells, which are transformed human fetal glial cells that express JCPyV large T antigen (TAg) (Daniel et al., 1996). Archetype JCPyV DNA also replicates in COS-7 cells, monkey cells that constitutively express SV40 TAg, but infectious progeny are not produced (Hara et al., 1998). Additionally, it has recently been shown that archetype JCPyV propagates in COS-7 cells stably expressing HIV-1 Tat after 32 days in culture (Nukuzuma et al., 2009). HIV-1 Tat acts in coordination with the cellular protein Pur α to stimulate viral DNA replication (Daniel et al., 2001). Importantly, these systems did not attain large scale propagation of archetype viruses. Therefore, in this study we sought to develop an improved culture system for archetype BKPyV and JCPyV.

We have previously shown that the BKPyV NCCR is the major genetic region that determines the ability of rearranged variants and not archetype virus to replicate viral DNA in RPTE cells (Broekema et al., 2010). We created a system that allowed us to exchange the major genetic regions between archetype and rearranged forms and showed that the rearranged NCCR present in an archetype virus background is sufficient for viral replication. It is also known that, in addition to the contribution of the NCCR, the early coding region product, TAg, is required *in*

trans for viral replication by binding to the viral origin of replication to initiate DNA synthesis (Fanning et al., 2009). Previous research has shown that SV40 TAg is capable of binding the origins of both JCPyV and BKPyV and initiating replication of their viral DNAs *in vitro* as well as *in vivo* (Daniel et al., 1996; Mahon et al., 2009; Sock et al., 1993). Additionally, BKPyV TAg and JCPyV TAg have some capacity to function in BKPyV, JCPyV, and SV40 DNA replication.

In this study we show that archetype BKPyV produces undetectable levels of TAg in cell culture models that support viral replication of rearranged variants, whose TAg production is robust. This knowledge, combined with data from previous studies, led us to ask if TAg overexpression could stimulate archetype BKPyV and JCPyV replication. We looked at the contribution of TAg transient overexpression by cotransfecting a BKPyV TAg cDNA with the viral genome and found that this could stimulate viral DNA replication and capsid protein production in archetype virus, which suggests progeny virus was produced. Therefore, we reasoned that constitutive high TAg levels may support archetype virus propagation. We chose to assess the contribution of TAg overexpression to viral replication in 293TT cells, human embryonic kidney cells constitutively expressing SV40 TAg (Buck et al., 2004), and successfully developed a means to efficiently propagate both BKPyV and JCPyV archetype virus for future study. Additionally, we determined whether SV40 TAg overexpression could stimulate the replication of other unculturable human polyomaviruses: Merkel cell polyomavirus (MCPyV), KI polyomavirus (KIPyV), and WU polyomavirus (WUPyV). 293TT cells were found to only propagate viruses closely related to SV40 whereas MCPyV, KIPyV, and WUPyV all failed to replicate efficiently in these cells.

Results

Archetype BKPyV produces undetectable level of TAg in RPTE cells. Rearranged forms of BKPyV are able to replicate in the natural host cell culture model RPTE cells, whereas archetype virus is unable to efficiently replicate in culture (Broekema et al., 2010; Hara et al., 1986; Watanabe et al., 1985). TAg is the viral protein responsible for initiating viral DNA synthesis as a result of binding to the origin of replication to recruit DNA polymerase (Fanning et al., 2009). Therefore, TAg protein production was first assessed in cells transfected with rearranged or archetype viral genomes to determine if the TAg level was a factor limiting archetype virus replication in RPTE cells. Recombinant archetype (Dik) and rearranged (Dunlop) BKPyV genomes were excised from the vector backbone, recircularized, and transfected into RPTE cells. We assayed for TAg production by collecting total cell proteins 4 dpt and immunoblotting for TAg protein (Figure 3.1A). TAg was only detectable when the rearranged genome, and not the archetype genome, was transfected into RPTE cells. Since TAg is necessary for viral DNA replication, we hypothesized that limited TAg expression from the archetype NCCR production was likely one key factor limiting the propagation of archetype virus in RPTE cells and that TAg overexpression, therefore, may be able to rescue archetype virus replication. Transient TAg overexpression in RPTE cells, by cotransfecting a BKPyV TAg cDNA with the archetype genome, did result in a detectable level of capsid protein production at 4 dpt (Figure 3.1B). Viral DNA replication was assessed in the same experiment by a *DpnI* resistance assay, which distinguishes methylated input plasmid DNA from unmethylated DNA that has been replicated in eukaryotic cells (Pipas et al., 1983). At 4 dpt, low molecular weight DNA was isolated, linearized, digested with *DpnI*, and assayed by Southern blotting. Replication was not

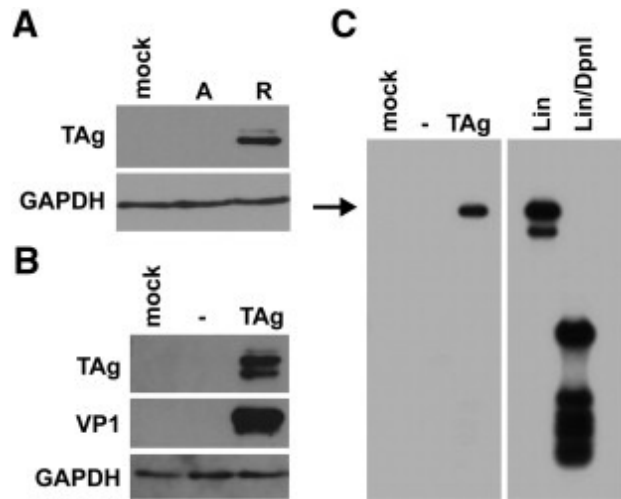


Figure 3.1. TAg expression limits archetype BKPyV replication in RPTE cells. A. RPTE cells were transfected with recircularized archetype (A) or rearranged (R) viral genomes. Total cell protein was harvested 4 dpt and 25 μ g of protein was analyzed by Western blotting for the expression of TAg and GAPDH. B. RPTE cells were transfected with recircularized archetype genome and empty vector (-) or BKPyV TAg cDNA. Total cell protein was harvested 4 dpt and 30 μ g was analyzed by Western blotting for the expression of TAg, VP1, and GAPDH. C. RPTE cells were transfected with recircularized archetype genome and empty vector (-) or BKPyV TAg cDNA, and low molecular weight DNA was harvested 4 dpt. Samples were linearized, digested with *DpnI*, and analyzed by Southern blotting. Arrow indicates the replicated viral genome. Input DNA is not visible on this exposure. Mock, mock transfection. Lin, linearized BKPyV plasmid control. Lin/*DpnI*, linearized and *DpnI*-digested BKPyV plasmid control. The blots shown are representative of three independent experiments.

detectable in control cells transfected with empty vector and the archetype genome (Figure 3.1C), consistent with previous results (Broekema et al., 2010). However, viral DNA replication was detectable when the archetype genome was cotransfected with BKPyV TAg cDNA. This demonstrates that TAg overexpression can stimulate archetype virus replication and capsid protein production.

Archetype BKPyV and JCPyV replicate efficiently and produce capsid protein in 293TT

cells. To determine whether sustained higher levels of TAg overexpression could promote robust archetype BKPyV replication, we performed viral replication assays in 293TT cells, human embryonic kidney cells that constitutively express high levels of SV40 TAg (Buck et al., 2004). 293TT cell contain an SV40 TAg expression plasmid in addition to an integrated SV40 genome which produces low levels of TAg due to a splicing bias that favors small t antigen. We reasoned that SV40 is a closely related virus and it has been shown that SV40 TAg can drive DNA replication from the origins of BKPyV and JCPyV (Daniel et al., 1996; Lynch et al., 1990; Mahon et al., 2009). Recombinant archetype and rearranged BKPyV genomes were excised from the vector backbone, recircularized, and transfected into 293TT cells. At 4 dpt, low molecular weight DNA was isolated, linearized, digested with *DpnI*, and assayed by Southern blotting (Figure 3.2A). Day 4 was chosen because rearranged variant replication can be easily detected at this time point (Broekema et al., 2010). Replication was quantified by normalizing the replicated genome band to a *DpnI*-digested input band, which controls for transfection efficiency. The quantified value of archetype virus DNA replication was set to 100. In two of four independent experiments, archetype virus replicated better than the rearranged virus while in the other two experiments there were almost equal levels of replication between the two viruses. When the data was averaged for all experiments, rearranged variant replication was 83.8% that of

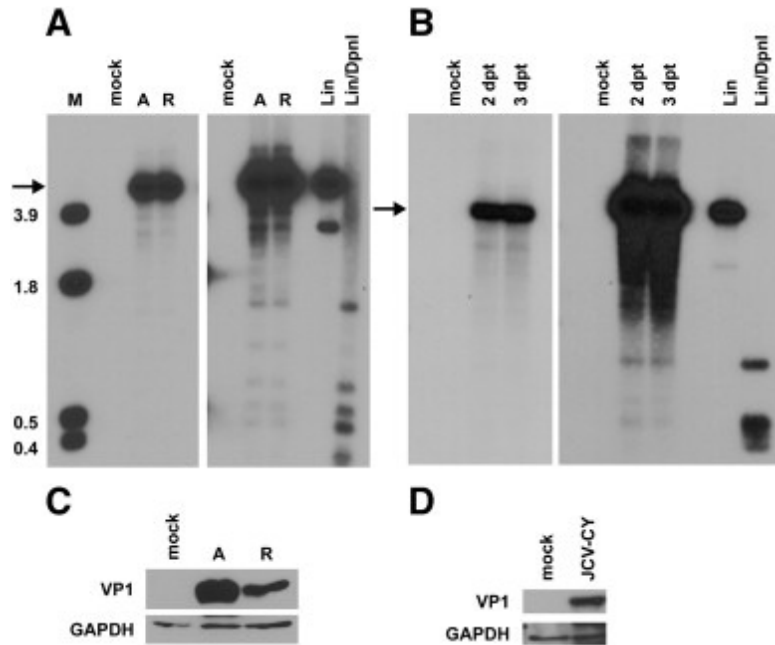


Figure 3.2. Archetype BKPyV and JCPyV replicate efficiently in 293TT cells. A. 293TT cells were transfected with recircularized archetype (A) or rearranged (R) BKPyV genomes, and low molecular weight DNA was harvested at 4 dpt. Samples were linearized, digested with *Dpn*I, and analyzed by Southern blotting. The right panel shows a darker exposure where the input *Dpn*I digested bands and digested plasmid controls are visible. Arrow indicates the replicated viral genome. M, *Hind*III digest of pGEM-TU (size of bands in kb). Lin, linearized BKPyV plasmid control. Lin/*Dpn*I, linearized and *Dpn*I-digested BKPyV plasmid control. B. Low molecular weight DNA was harvested 2 and 3 dpt from archetype JCPyV transfection and analyzed by Southern blotting. Light and dark exposures are shown as in A. C, D. Analysis of VP1 expression. C. Total cell protein was harvested from 293TT cells 4 dpt and 90 μ g of protein was analyzed by Western blotting for BKPyV VP1 and GAPDH expression. Mock, mock transfection. D. Total cell protein was harvested 4 dpt and 60 μ g of protein was analyzed by Western blotting for JCPyV VP1 and GAPDH expression. The blots shown are representative of three independent experiments.

archetype virus with a standard deviation of 29.8%. This was not statistically different from that of archetype virus (Student's t test p value of 0.3). Therefore, both archetype and rearranged BKPyV replicated well in 293TT cells. This is in contrast to RPTE cells, in which rearranged BKPyV replicates ~75 fold better than archetype (Broekema et al., 2010). Archetype JCPyV replication was also assessed at 2 and 3 dpt by *DpnI* resistance assay (Figure 3.2B). Archetype JCPyV efficiently replicated in 293TT cells, and somewhat faster than BKPyV. We also measured capsid protein production in 293TT cells for archetype BKPyV and JCPyV by collecting total cell proteins 4 dpt and Western blotting for VP1 protein. Consistent with the replication assay results, archetype BKPyV and JCPyV produced capsid protein in 293TT cells (Figure 3.2C and D). Additionally, archetype BKPyV appeared to produce more VP1 than rearranged. Encouraged by the results with these archetype viruses, we assessed capsid protein production and replication of three other unculturable human polyomaviruses: MCPyV, KIPyV, and WUPyV. We obtained capsid protein antibody for KIPyV and WUPyV and these viruses did not produce detectable levels of capsid protein as assayed by immunoblotting (Figure 3.3A and B). Capsid production was not tested for MCPyV due to lack of an available antibody. These viruses were not capable of significant replication as measured by a *DpnI* resistance assay (Figure 3.3C-E). These results show that archetype BKPyV and JCPyV but not MCPyV, KIPyV, and WUPyV are able to replicate their DNA and express late proteins in 293TT cells.

Infectious progeny production of archetype BKPyV and JCPyV. Production of VP1 by archetype virus suggests that viral progeny are also produced in 293TT cells. To test whether archetype virus was in fact producing infectious progeny virus we harvested whole cell lysates 7 dpt. We then infected fresh 293TT cells with 1:2 dilutions of the transfection lysate in serum-free

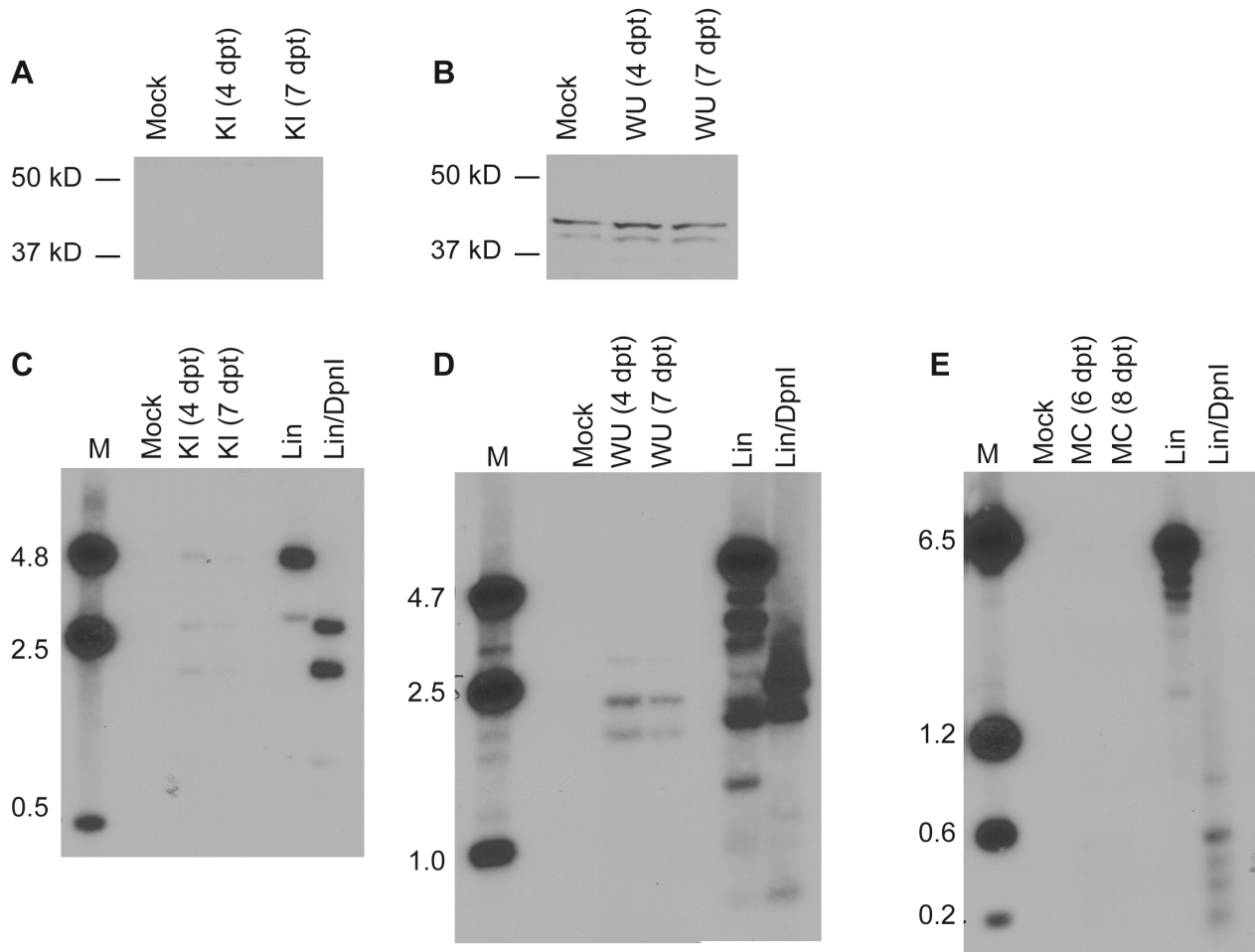


Figure 3.3. KI, WU, and MCPyV do not replicate efficiently in 293TT cells. 293TT cells were transfected with recircularized viral genomes. Total cell protein was harvested from 293TT cells 4 and 7 dpt and 80 μ g of protein was analyzed by Western blotting for A. KIPyV VP1 (predicted 45 kD). B. WUPyV VP1 (predicted 44 kD). Low molecular weight DNA was harvested at the indicated timepoint. Samples were linearized, digested with *DpnI*, and analyzed by Southern blotting. C. KIPyV transfection analyzed by Southern blot. M, *PciI* digest of pBlu-KI (size of bands in kb). Lin, linearized KIPyV plasmid control. Lin/*DpnI*, linearized and *DpnI*-digested KIPyV plasmid control. D. WUPyV transfection analyzed by Southern blot. M, *KpnI* digest of pBlu-WU (size of bands in kb). Lin, linearized WUPyV plasmid control. Lin/*DpnI*, linearized and *DpnI*-digested WUPyV plasmid control. E. MCPyV transfection analyzed by Southern blot. M, *XbaI* digest of pMCV-R17a (size of bands in kb). Lin, linearized MCPyV plasmid control. Lin/*DpnI*, linearized and *DpnI*-digested MCPyV plasmid control. Mock, mock transfection. The blots shown are representative of two or three independent experiments.

medium and assessed capsid protein production over time as a measure of infection. We could not use BKPyV or JCPyV TAg protein expression as a readout of infection because of the abundant SV40 TAg expression in 293TT cells, which is detected by the SV40 TAg pAb416 antibody. Therefore, VP1 capsid protein was assessed at 1, 4, and 7 days post infection (dpi). Archetype BKPyV lysates showed increased capsid protein production over time in 293TT cells. Consistent with the transfection DNA replication and capsid protein results, archetype virus appeared to have somewhat accelerated kinetics of infection when compared to the rearranged variant (Figure 3.4A). Infection on fresh 293TT cells with 7 dpi lysates of archetype JCPyV also resulted in an increased VP1 protein over time (Figure 3.4B). These results show that archetype BKPyV and JCPyV are able to produce infectious progeny in 293TT cells. Lysates harvested from cells transfected with the KIPyV and WUPyV genomes were unable to produce infectious progeny as measured by lack of capsid protein production when passaged on fresh 293TT cells. This result is consistent with the inability of these viruses to replicate DNA in 293TT cells.

Previously, it has been shown that rearrangements in the NCCR of archetype BKPyV can occur when it is passaged in cell culture (Rubinstein et al., 1991). Therefore, the integrity of the regulatory region of the virus in the lysates used for infection was confirmed by PCR amplifying the regulatory region from one transfection lysate, cloning the PCR products, and sequencing. Three clones from the rearranged BKPyV lysate were sequenced and no mutations were found. Nine clones from the archetype BKPyV lysate also had no rearrangements or point mutations. Ten clones were sequenced from the JCPyV regulatory region, none of which contained any rearrangements. Three of the ten, however, did contain base changes, at G108A, G235A, and C253T, where 1 is the first nucleotide of the regulatory region. Single nucleotide changes are not

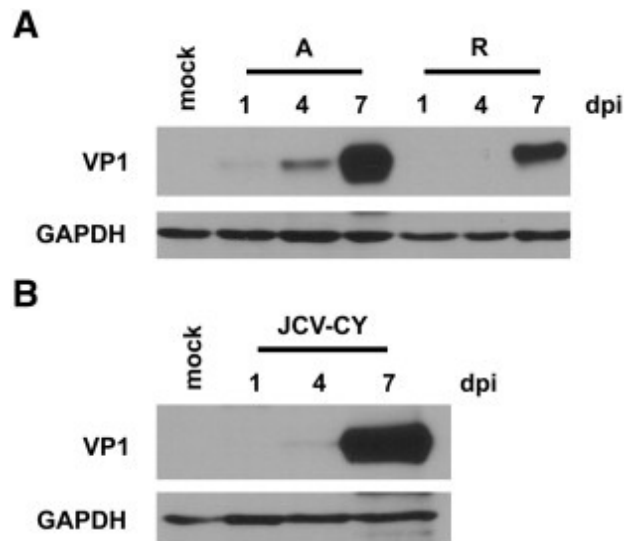


Figure 3.4. Archetype BKPyV and JCPyV produce infectious progeny in 293TT cells. Cell lysates were harvested from 293TT cells 7 dpi. The lysates were then used to infect fresh 293TT cells. A. Total cell protein was harvested on 1, 4, and 7 dpi with BKPyV lysate, and 50 μ g of protein was analyzed by Western blotting for BKPyV VP1 and GAPDH expression. A, archetype. R, rearranged. B. Total cell protein was harvested on 1, 4, and 7 dpi with JCPyV lysate, and 50 μ g of protein was analyzed for JCPyV VP1 and GAPDH expression. The blots shown are representative of three independent experiments.

rearrangements and therefore all archetype BKPyV and JCPyV clones sequenced maintained an archetype regulatory region structure.

Purification of archetype BKPyV from 293TT cells and virion morphology. We next examined the physical properties of the BKPyV produced in 293TT cells. Archetype and rearranged BKPyV from 293TT cells were purified on a CsCl gradient. The refractive index of the mature virus band for both forms was measured and the density was calculated to be 1.34g/cm^3 , the expected polyomavirus density. Purified virus morphology was then examined by transmission electron microscopy (TEM) (Figure 3.5). As a comparison, infectious rearranged (TU) BKPyV viral particles from Vero cells, which support rearranged variant replication, are shown. The average sizes of the archetype virions were 44.9 ± 2.0 nm ($n=85$), rearranged virions were 46.6 ± 2.5 nm ($n=34$), and particles purified from Vero cells were 46.4 ± 1.4 nm ($n=36$). The number of virus particles observed on the grids was roughly equivalent between the viruses purified from 293TT and Vero cells, indicating equivalent progeny production.

To examine the integrity of the NCCR of the purified virus, the NCCR was PCR amplified and cloned, and ten clones were sequenced from each virus. No rearrangements were identified in the archetype virus NCCR, but one clone from the rearranged variant had a deletion of the first two P blocks of the Dunlop NCCR. Single nucleotide changes were identified in each form, in 3 of 10 clones from the rearranged variant and 1 of 10 clones from the archetype virus. The rearranged variant base substitutions were T28C, A46G, and T322C, where 1 is the first nucleotide of the O block. The archetype virus substitution was C141T, which maps to the BKPyV TAg binding site III. However, this substitution does not make the site more similar to that of SV40 TAg binding site III, which may be expected if selection for use of SV40 TAg were occurring.

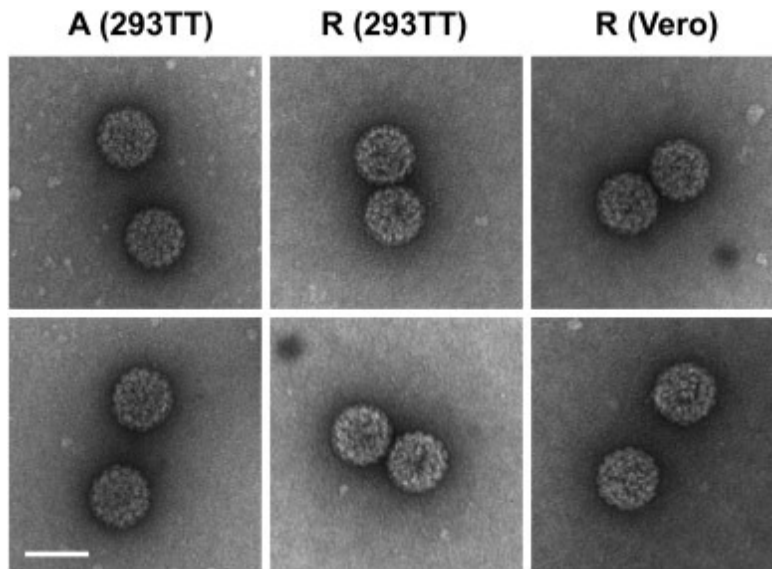


Figure 3.5. Morphology of BKPyV produced in 293TT cells. Virus was purified from cells by CsCl gradient centrifugation. Mature virus particles were applied to grids as described in the text. Viral particles were visualized by negative staining at a magnification of 180,000 \times . Archetype virions from 293TT cells are shown in the left column, rearranged virions from the same cells in the middle column, and rearranged virions purified from Vero cells in the right column. Scale bar = 50 nm. The particles shown are representative of three independent purifications.

Purified virus was treated with DNase to digest any unencapsidated viral DNA and progeny virions were quantified by real-time PCR. In a representative experiment, archetype virus purified from 293TT cells had 2.84×10^{11} genomes/ml and rearranged virus had 1.65×10^{11} genomes/ml. Infectivity of the purified virus was confirmed by infecting 293TT cells with 500 genomes/cell and measuring VP1 expression at 1, 3, and 5 dpi (Figure 3.6). Both purified archetype and rearranged viruses were able to infect 293TT cells and showed an increase in capsid protein over the five day time course. We noted no difference in viral kinetics when cells were infected with equal genome numbers. Next we estimated the amount of infectious virus present by infecting 293TT cells and immunostaining for VP1. As a control to validate the assay, rearranged virus purified from Vero cells was quantified by both VP1 immunostaining in 293TT cells and TAg immunostaining in RPTE cells, a standard infectious unit (IU) assay; it was calculated to have an equal titer in both assays. In the representative experiment of virus whose genomes were quantified above, the viral titers for archetype and rearranged virus were measured to be 2.14×10^8 and 2.40×10^7 IU/ml, respectively. In comparison, the yield from rearranged virus grown in Vero cells in three fold more cells is, on average, 10^8 IU/ml. Archetype and rearranged virus purified from 293TT cells therefore have a ratio of 1352 and 6875 genomes/IU, respectively. This result was reproducible. Taken together, these results show that archetype BKPyV purified from 293TT cells is infectious and that the yields of infectious virus are similar to those obtained with rearranged variants in Vero cells.

Discussion

BKPyV and JCPyV cause severe clinical disease in immunosuppressed and immunocompromised patients, respectively. It is thought that the archetype form of these viruses

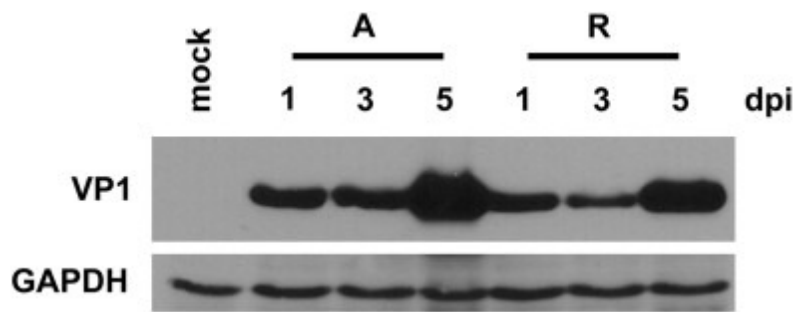


Figure 3.6. Purified archetypal BKPyV is infectious in 293TT cells. 293TT cells were infected with 500 genomes/cell of purified BKPyV and total cell protein was harvested 1, 3, and 5 dpi. 50 μ g of protein was analyzed by Western blotting for BKPyV TAg and GAPDH expression. A, archetypal. R, rearranged. The blots shown are representative of three independent experiments.

is acquired in childhood or early adulthood and establishes a persistent infection in the host. Thus far, cell culture systems to propagate archetype polyomaviruses have been very limited in their availability and robustness. Prior to this study we noted that archetype BKPyV replicated very poorly in RPTE cells, and we showed here that when RPTE cells were transfected, the archetype virus genome produced undetectable levels of TAg protein. Since TAg is required to initiate viral DNA synthesis, we hypothesized that overexpression of TAg would stimulate archetype virus replication. We show here that transient BKPyV TAg overexpression with archetype BKPyV transfection is able to rescue replication as assayed by capsid protein production. Additionally we show that 293TT cells, which greatly overexpress SV40 TAg, are capable of propagating both archetype BKPyV and JCPyV. These viruses were able to replicate their DNA, express VP1, and produce infectious progeny that were capable of reinfecting 293TT cells. This represents an improved culture model system for archetype BKPyV and JCPyV.

In addition to testing replication of archetype BKPyV and JCPyV, we also assessed three other unculturable polyomaviruses: MCPyV, KIPyV, and WUPyV. Each of these viruses showed only minimal replication in 293TT cells as assessed by the *DpnI* resistance assay, akin to what is seen for archetype BKPyV in RPTE cells (Broekema et al., 2010). Additionally, KIPyV and WUPyV were unable to express detectable levels of capsid protein. The TAg coding sequences of MCPyV, KIPyV, and WUPyV have only a 47.6%, 64.4%, and 62.8% similarity to SV40 TAg, respectively, whereas BKPyV has 85.9% and JCPyV has 84.1% similarity. Our results suggest that SV40 TAg is likely not similar enough to bind to and initiate replication from the MCPyV, KIPyV, and WUPyV origins of replication.

It has been shown previously that SV40 TAg is capable of initiating replication from both the archetype and rearranged JCPyV and BKPyV origins (Daniel et al., 1996; Mahon et al.,

2009; Sock et al., 1993). BKPyV TAg and JCPyV TAg also have some capacity to initiate replication of BKPyV, JCPyV, and SV40 DNA, although replication of SV40 DNA initiated by JCPyV TAg is limited. Our results show robust replication of JCPyV at early timepoints post transfection of 293TT. This is consistent with previous results showing replication of JCPyV DNA initiated by SV40 TAg is better than that of BKPyV DNA (Mahon et al., 2009). Archetype JCPyV DNA can replicate but not efficiently produce virus in POJ cells and COS-7 cells overexpressing JCPyV and SV40 TAg, respectively (Daniel et al., 1996; Hara et al., 1998; Nukuzuma et al., 2009). The difference in progeny production of archetype JCPyV from COS-7 cells compared to 293TT cells likely has to do with the very high levels of SV40 TAg produced in 293TT cells. Furthermore, archetype BKPyV grown in 293TT cells is able to produce as many IU/ml as rearranged virus grown in Vero cells, indicating efficient propagation. In contrast, previous research in a human endothelial cell line, archetype BKPyV infectious progeny production was poor and not detected until 35 dpt (Hanssen Rinaldo et al., 2005).

Previous attempts to propagate archetype BKPyV in human embryonic kidney cells resulted in selection for rearrangements (Rubinstein et al., 1991). It also appears that significant rearrangement of the NCCR was selected for upon propagation in endothelial cells (Hanssen Rinaldo et al., 2005). Therefore, we confirmed the integrity of the regulatory regions of the viruses produced in 293TT cells. Single nucleotide changes were observed in a minority of the clones sequenced. These may be bona fide mutations or possibly PCR errors, although we did use high fidelity Taq polymerase . However, single base changes have been shown to be common in archetype BKPyV and are not likely to affect replication (Yogo et al., 2008). There was only one major rearrangement identified in one clone from the BKPyV rearranged NCCR, which was a deletion of two of three P blocks. However, no rearrangements were observed in the

archetype virus regulatory region of BKPyV and JCPyV. These results suggest that there is no selective pressure in 293TT cells to induce rearrangements which might facilitate replication. The TEM images of the purified BKPyV support the identity of the purified virus as authentic polyomavirus.

Archetype JCPyV DNA replicates in COS-7, simian kidney cells expressing SV40 TAg, and COS-7 cells expressing HIV-1 Tat (Hara et al., 1998; Nukuzuma et al., 2009). However, these systems show weak progeny production, taking up to 32 days to produce JCPyV as detected by hemagglutination assay. Our system has the advantage of faster progeny production, since we detect significant titers, measured by infecting fresh 293TT cells with lysate harvested 7 dpt. 293TT cells are a human cell line and thus can be more effectively used to study unique host-virus interactions that may be present, such as interactions between cellular transcription factors and archetype virus promoters. However, since archetype virus replication in 293TT cells is driven by SV40 TAg, these cells cannot be used as a natural host cell to study the life cycle of polyomaviruses. Regardless, these cells will allow for further detailed comparative study of archetype polyomaviruses by providing a means for their propagation in the laboratory.

In conclusion, 293TT cells are able to support growth of the closely related archetype BKPyV and JCPyV. Together, these findings strongly suggest that TAg production is a limiting factor for archetype polyomavirus propagation in currently-available cell culture models. Based on the results of this study, we speculate that cell culture systems overexpressing TAg from other unculturable polyomaviruses may be able to support propagation of those viruses. The findings in this paper expand our understanding of archetype virus and are an early step toward characterizing the differences between archetype and rearranged polyomaviruses. This study and

knowledge gained from future studies may lead to new approaches to prevent transmission or reactivation of archetype virus in healthy individuals and immunocompromised hosts.

Materials and Methods

Cell culture. Primary human RPTE cells were maintained for up to six passages in renal epithelial growth medium as previously described (Abend et al., 2007). 293TT cells were obtained from Chris Buck at the National Cancer Institute (Buck et al., 2004) and were maintained under 400 µg/ml hygromycin selection in DMEM with 10% fetal bovine serum and 100 U/ml penicillin, 100 µg/ml streptomycin.

Plasmids. The pBR322-Dunlop plasmid (ATCC #45025, pBKV) contains a full length Dunlop (rearranged NCCR) genome (Seif et al., 1979) and the pBR322-Dik plasmid contains a full length Dik (archetype NCCR) genome (Nishimoto et al., 2006). The Dik and Dunlop genomes each were subcloned from these plasmids into pGEM7 at the *Bam*HI site to facilitate plasmid growth. The pJC-CY plasmid containing archetype JCPyV genome cloned into pUC19 at the *Bam*HI (Yogo et al., 1990) was received from Richard Frisque at Pennsylvania State University. The pBlu-WU and pBlu-KI plasmids were received from David Wang at Washington University. We obtained pMCV-R17a (Schowalter et al., 2010) from Addgene (plasmid 24729), where it had been deposited by Chris Buck. The BKPyV TAg cDNA plasmid was engineered for other purposes with a silent mutation at nucleotide 4406 (Harris et al., 1998). For the present study, the cDNA was subcloned into pcDNA3.1(+)-hygro (Invitrogen) using the *Bam*HI and *Eco*RI sites.

Transfection. The viral genome was excised from the vector backbone by *Bam*HI or *Eco*RI digestion (New England Biolabs, Ipswich, MA) and then recircularized at a concentration of 10 ng/µL using T4 DNA ligase (New England Biolabs). RPTE or 293TT cells were seeded into 12

well plates and transfected with 0.5 µg DNA at 70-80% confluency using TransIT LT-1 transfection reagent (Mirus Bio, Madison, WI) according to the manufacturer's instructions, with a DNA to transfection reagent ratio of 1:6. For the TAg cotransfection experiments, 0.5 µg of total DNA was transfected at a ratio of 1:9 of TAg to viral genome. Transfection complexes were removed 18 hr post-transfection by washing once with PBS and adding fresh media. DNA was extracted from cell lysates by phenol-chloroform extraction, and the integrity of the regulatory regions of BKPyV and JCPyV was confirmed in transfection lysates 7 dpt by cloning PCR products of the BKPyV NCCR and JCPyV regulatory region (RR) into pGEM using primers BKPyV NCCR Forward (5' TTTGTCAGGGTGAAATTCCTT 3') and BKPyV NCCR Reverse (5' TCACCTCTACAAAATTCCAGCA 3') and JCPyV RR Forward (5' GAATGTTTCCCCATG CAGAT 3') and JCPyV RR Reverse (5' CTGTGCAAAAAGTCCAGCAAAA 3'). The PCR reaction consisted of 0.5 µM each primer, 1 mM dNTPs, 2 mM MgSO₄, and 1 U Platimun *Tag* High Fidelity polymerase (Invitrogen) in 1X buffer provided by the manufacturer. The PCR program consisted of an initial 5 min denaturation at 95°C followed by 30 cycles each of denaturation at 95°C for 45 sec, annealing at 55°C for 1 min, and elongation at 68°C for 1 min. The PCR product was purified using the QIAquick PCR Purification Kit (Qiagen), ligated into pGEM-T Easy vector (Promega), and clones were sequenced.

Southern Blotting. *DpnI* resistance assays were performed as previously described (Broekema et al., 2010) using low molecular weight DNA harvested at the times stated in the text. For BKPyV, the probe was a 3239 bp *PvuII* fragment excised from pGEM7-TU (Abend et al., 2007) which spans the early region and the NCCR. The size marker was pGEM7-TU digested with *HindIII*. For JCPyV, the probe was the whole 5120 bp JCPyV-CY genome excised from pJC-CY by *BamHI* digestion. For KIPyV, the probe was the whole KIPyV genome excised from the

pBlu-KI plasmid by *EcoRI* digestion. The marker was pBlu-KI digested with *PciI*. For WUPyV, the probe was the whole WUPyV genome excised from the pBlu-WU plasmid by *EcoRI* digestion. The marker was pBlu-WU digested with *KpnI*. For MCPyV, the probe was the whole MCPyV genome excised from the pMCMV-R17a plasmid by *EcoRI* digestion. The marker was pMCMV-R17a digested with *XbaI*.

Western Blotting. E1A lysis buffer was used to harvest total cell proteins, which were analyzed as previously described (Jiang et al., 2009b) with the following modifications. The membrane was blocked for 30 min at room temperature (RT) in 2% nonfat dried milk in PBS with 0.1% Tween 20 (PBS-T), then incubated with primary antibody in 2% nonfat dried milk in PBS-T for 2 hr at RT or overnight at 4°C. The following antibodies and concentrations were used: SV40 pAb416 (Harlow et al., 1981) for TAg at 1:3,000; P5G6 (received from D. Galloway) for BKPyV VP1 at 1:10,000; pAb597 (Atwood et al., 1995) (received from R. Frisque) for JCPyV VP1 at 1:5,000; WUPyV VP1 and KIPyV VP1 antibodies at 1:1,000 (Nguyen et al., 2009) (received from D. Wang); and Ab9484 (Abcam) for GAPDH at 1:10,000. The membrane was then washed 3 x 10 min with PBS-T and incubated with horseradish peroxidase-conjugated sheep anti-mouse antibody (Amersham) at 1:5,000 in 2% nonfat dried milk at room temperature for 2 hr. The membrane was washed 3 x 10 min and developed using HyGLO (Denville Scientific Inc.) or Luminol (Millipore).

Virus Purification. Virus purification was performed by density centrifugation on CsCl gradients as previously described (Jiang et al., 2009b). The mature virus bands were collected by gravity flow from the side of the centrifuge tube using an 18-gauge needle. The collected virus was dialyzed against buffer A (10mM HEPES [pH7.9], 1mM CaCl₂, 1mM MgCl₂, 5 mM KCl) overnight at 4°C. The density of each band was determined by measuring the refractive index.

The integrity of the NCCR was confirmed by cloning PCR products of the BKPyV NCCR using primers BKPyV NCCR Forward and BKPyVV NCCR Reverse as described above and sequencing 10 clones for each. Purified virus was DNase-treated to digest unencapsidated viral DNA and progeny genomes were quantified by real-time PCR as previously described (Jiang et al., 2011) using primers TAg-Forward (5' TGTGATTGGGATTCAGTGCT 3') and TAg-Reverse (5' AAGGAAAGGCTGGATTCTGA 3').

Infection. 293TT cells were infected at 70-80% confluency with 500 genomes/cell of purified archetype or rearranged virus, or crude viral lysate, in low serum media at 4°C. After 1 hr the infection media was replaced with fresh warmed maintenance media. Viral titer was determined by an infectious unit assay. Fifty percent confluent 293TT cells were infected at 4°C with 10 fold dilutions of purified virus. Infection was allowed to proceed for 3 dpi at 37°C. The cells were then fixed for 5 min in 95% ethanol/5% acetic acid and stained with the monoclonal anti-VP1 antibody P5G6 (received from D. Galloway) at 1:500 followed by a fluorescein-conjugated anti-mouse antibody at 1:100. Viral titer was determined by averaging five fields of view in triplicate infections.

Transmission Electron Microscopy. Dialyzed virus was diluted 1:10 in buffer A and applied to glow-discharged 300-mesh copper grids (Electron Microscopy Sciences) for 5 min at RT. Grids were then washed in buffer A, fixed with glutaraldehyde, and stained with uranyl acetate as previously described (Christensen et al., 2008).

Sequence alignment. TAg coding sequences were aligned by pairwise sequence alignment (<http://www.ebi.ac.uk/Tools/psa/>).

Notes

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Chapter IV

miRNA regulation of BK polyomavirus replication during early infection

Abstract

Viral microRNAs (miRNAs) play an important role during infection by post-transcriptionally regulating both host and viral gene expression. However, the function of many viral miRNAs remains poorly understood. In this study, we investigated the role of the BK polyomavirus (BKPyV) miRNA in regulating virus replication. The function of the polyomavirus miRNA was investigated in an archetype BKPyV, which is the transmissible form of the virus and thought to establish a persistent infection in the host urinary tract. In agreement with previous studies, we show that the BKPyV miRNA targets early transcripts including large T antigen (TAg) mRNA. Importantly, we show that the miRNA plays a significant role in limiting archetype BKPyV replication in a natural host cell model of infection. This regulation is accomplished through the balance of promoter activities controlling early gene expression and miRNA expression prior to genome replication. We therefore provide evidence for a novel function of the polyomavirus miRNA that may have important implications for the mechanism of viral persistence.

Introduction

microRNAs (miRNAs) are small non-coding RNAs that post-transcriptionally regulate gene expression by repressing translation or directing cleavage of target mRNAs (Bartel, 2004).

miRNAs regulate diverse cellular processes and are generally considered fine tuners of gene expression (Baek et al., 2008). In animal cells, miRNAs usually recognize targets with imperfect complementarity, therefore, an individual miRNA may have hundreds of targets. However in plants and some viruses, miRNAs are able to recognize a target with perfect complementarity and direct cleavage of the target in a manner similar to small interfering RNAs (siRNAs). Virally-encoded miRNAs have been described that target host gene expression, viral gene expression, or both. However, the function of many viral miRNAs remains poorly understood. Viruses such as herpesviruses autoregulate their gene expression through the use of miRNAs (Gottwein et al., 2008), and autoregulation of viral genes has been implicated in regulating viral latency or persistence.

Polyomaviruses are species-specific viruses with small double-stranded DNA genomes. BK polyomavirus (BKPyV) is the causative agent of polyomavirus-associated nephropathy (PVAN) and hemorrhagic cystitis (HC) in kidney transplant and bone marrow transplant recipients, respectively (Ahsan et al., 2006). There are currently no FDA-approved drugs to treat BKPyV-associated diseases. There are two forms of the BKPyV genome, designated archetype virus and rearranged variants. These forms are distinguished by the DNA sequence of their non-coding control region (NCCR). The archetype virus is thought to be the transmissible form of the virus, since it is found in both healthy people, in which the virus establishes a persistent subclinical infection, and in diseased patients. The NCCR structure of archetype virus is divided into five sequence blocks termed O, P, Q, R, and S (Moens et al., 2005) (Figure 4.1). The O block is named for the origin of replication; while the P, Q, R, and S blocks are the enhancer region and contain transcription factor binding sites for the divergent early and late promoters. The early promoter drives the early coding region products TAg, small t antigen, and truncated T

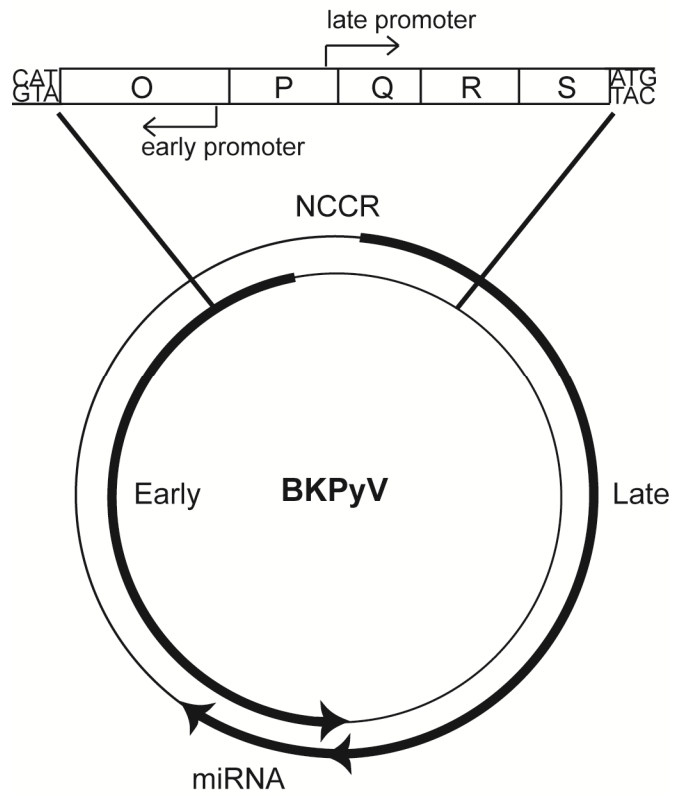


Figure 4.1. Schematic of the BKPyV genome. The viral genome is a double stranded circular DNA molecule. The early primary transcript (clockwise bold arrow) and the late primary transcript (counterclockwise bold arrow) are divergently transcribed from the NCCR by the early and late promoters, respectively. The miRNA is complementary to the early coding region and transcribed from the late strand.

antigen, while the late promoter drives the late coding region products agnoprotein and structural proteins VP1, VP2, and VP3. Rearranged variants are characterized by duplications and deletions of the blocks of sequence that make up the NCCR, and are associated with BKPyV disease (Gosert et al., 2008a). Rearranged variants, but not archetype virus, replicate in renal proximal tubule epithelial (RPTE) cells, a natural cell culture model of lytic replication (Low et al., 2004). We have previously shown that archetype virus produces undetectable levels of TAg and very low, if any, viral DNA replication in RPTE cells (Broekema et al., 2012). Therefore, there is currently no natural cell culture model of archetype virus replication.

Many polyomaviruses including SV40, murine polyomavirus (MPyV), JC polyomavirus (JCPyV), BKPyV, and Merkel cell polyomavirus (MCPyV) encode a miRNA that targets early coding region products, such as large TAg mRNA (Seo et al., 2009; Seo et al., 2008; Sullivan et al., 2005; Sullivan et al., 2009). One of the major functions of TAg is driving DNA replication of the viral genome at the origin of replication (Fanning et al., 2009). A conserved functional role of the polyomavirus miRNA autoregulation of TAg remains unclear, however. The SV40 miRNA was the first polyomavirus miRNA described, and was shown to downregulate early viral gene expression (Sullivan et al., 2005). Cells infected with an SV40 mutant lacking the pre-miRNA are more sensitive to TAg-specific cytotoxic T cell (CTL) lysis *in vitro*, suggesting that the miRNA is important in viral evasion of the host immune response. However, the miRNA mutant SV40 produces the same amount of infectious progeny as wildtype (wt) virus. A naturally occurring deletion mutant of the MPyV miRNA has no defects with respect to replication, transformation efficiency, or establishment or clearance of infection *in vivo* (Sullivan et al., 2009). Consequently, the miRNA was deemed to be non-essential for infection *in vitro* and *in vivo*. Unlike the SV40 miRNA, the MPyV miRNA does not limit the host CTL response

during either the acute or persistent phases of *in vivo* infection. Therefore, no clearly defined role, conserved among all polyomavirus miRNAs, has yet been described.

JCPyV and BKPyV encode one pre-miRNA on the late strand with perfect complementarity to the 3' coding end of the early mRNA (Figure 4.1) (Seo et al., 2008). The miRNA may be expressed from the late promoter, since it is located on the late strand, although it is possible that it has a unique promoter located within or outside the NCCR. In animal cells, normally one arm of the miRNA duplex is preferentially loaded into the RNA-induced silencing complex (RISC) while the other arm is degraded (Bartel, 2004). However, modified 5' RACE analysis showed that both arms of the BKPyV and JCPyV miRNA direct degradation of the viral TAg mRNA target, in an siRNA-like fashion (Seo et al., 2008). No consequence of TAg downregulation has yet been identified for the JCPyV or BKPyV miRNAs. However, since TAg is responsible for initiating viral DNA replication (Fanning et al., 2009), we reasoned that the miRNA may play a role in limiting viral replication. While previous studies with SV40 and MPyV have used only rearranged variants, we were interested in evaluating the functional role of the miRNA in the archetype form of the virus, which establishes a persistent infection in the urinary tract of the host (Heritage et al., 1981b).

This is the first study examining the role of a polyomavirus miRNA using an archetype virus. While previous studies have indicated a role for the polyomavirus miRNA in limiting host immune system recognition, we show for the first time that a polyomavirus miRNA controls viral replication early in the course of infection. Since the miRNA is expressed from the late strand, it was assumed to autoregulate early viral gene expression late in the viral infection (Seo et al., 2008). However, we provide evidence that the miRNA can regulate early mRNA expression early, prior to genome replication. This regulation is accomplished through a delicate

balance of promoter activities controlling early gene expression and miRNA expression. We also provide evidence that the miRNA may have a unique promoter within the NCCR that fires in the late direction. In the present study, we define a novel role for the polyomavirus miRNA in regulating viral replication that is unique to the form of the virus that establishes persistence in the human host. This work implicates the miRNA as an important viral factor in the mechanism of polyomavirus persistence.

Results

A BKPyV miRNA mutant virus is unable to regulate its target early mRNA sequence.

BKPyV expresses a miRNA that targets its early mRNAs in an siRNA-like manner (Seo et al., 2008). To examine the role of the miRNA in limiting viral replication, we created BKPyV mutant viruses in the archetype and rearranged backgrounds that are unable to express both the mature 5 prime (5P) and 3P miRNAs (Figure 4.2B). Since the miRNA is complementary to the 3' end of the TAg coding sequence, it was necessary to mutate the miRNA sequence at wobble positions in the TAg coding sequence to conserve TAg function. Previously, an SV40 miRNA mutant was created by mutating a majority of the wobble positions in the pre-miRNA sequence, a total of 18 point mutations (Sullivan et al., 2005). We chose to create a BKPyV archetype virus miRNA mutant with fewer point mutations to minimize any potential off-target effects due to the mutations. Three positions in the pre-miRNA sequence were mutated, based on the predicted creation of a bubble in the pre-miRNA folded structure that would interfere with miRNA processing (Figure 4.2A). The wt and miRNA mutant viruses were purified from 293TT cells, which allow both archetype virus and rearranged variants to replicate (Broekema et al., 2012).

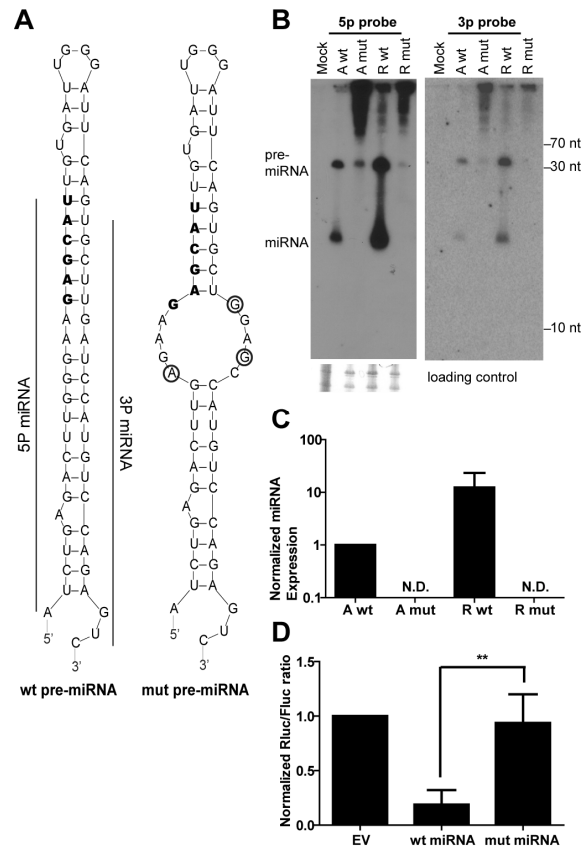


Figure 4.2. The BKPvV miRNA mutant is unable to regulate its target early mRNA. (A) Predicted fold of the wt and the mutant (mut) pre-miRNAs (29). Point mutations are indicated by circles and bases recognized by the stem-loop RT primer are in bold (B) The BKPvV miRNA mutant virus does not produce mature miRNA. 293TT cells were infected with purified wt or miRNA mutant virus at an MOI of 0.01. Total cell RNA was harvested 5 dpi. Mature 5P and 3P miRNAs were detected by Northern blotting. The blot was hybridized using the 5P probe (which recognizes the 5P arm), stripped, then re-hybridized using the 3P probe. Ethidium-stained rRNA bands are shown as a loading control. (C) BKPvV 5P miRNA expression was quantified by stem-loop RT qPCR and normalized to the cellular control hsa-let-7a. The A wt sample was arbitrarily set to 1. No miRNA expression was detected in mock, mock infected; A, archetype; R, rearranged; wt, wt miRNA; mut, mutant miRNA; N.D., not detected. (D) Relative luciferase levels from 293 cells co-transfected with the luciferase reporter plasmid and a plasmid expressing the wt BKPvV miRNA, mutant BKPvV miRNA, or empty vector (EV). *Renilla* luciferase (Rluc) values were normalized to firefly luciferase (Ffluc) values as a transfection control. Each bar is the average from four independent experiments and the error bars are standard deviation. N.D., not detected; **, $p < 0.01$. (The two-tailed, unpaired Student's t-test was performed for statistical analyses).

RNA was harvested from infected 293TT cells and Northern blotting confirmed that the mutant virus does not produce its mature 5P and 3P miRNAs (Figure 4.2B). To ensure that the wild type probe can recognize the mutant miRNA sequence despite the presence of the mutations, we probed a Southern blot containing plasmid DNAs corresponding to the wild type and mutant genomes, and saw no difference in hybridization (Figure 4.3). Additionally the mature 5P miRNA, the more abundant arm (Seo et al., 2008), was not detected in the mutant virus by stem-loop qPCR of RNA (Figure 4.2C), a more sensitive detection method (Figure 4.4).

To confirm the role of the BKPyV miRNA in targeting early mRNA, we used a luciferase assay to quantify the miRNA inhibitory activity (Seo et al., 2008). Plasmids expressing the wild-type (wt) or mutant BKPyV miRNAs were cotransfected into 293 cells with a plasmid encoding the miRNA target sequence cloned into the 3' untranslated region (UTR) of the *Renilla* luciferase gene. The cotransfection of the target plasmid and the BKPyV wt miRNA plasmid resulted in a significant decrease in luciferase levels compared to the miRNA mutant (Figure 4.2D). These results demonstrate that the BKPyV miRNA mutant is functionally unable to recognize the early mRNA target sequence.

Archetype BKPyV replication is limited by the viral miRNA. No previous study of polyomavirus miRNAs showed a role in regulating replication; however, all previous studies were performed using rearranged variants as opposed to archetype virus (Seo et al., 2008; Sullivan et al., 2005; Sullivan et al., 2009). To examine the role of the BKPyV miRNA in archetype virus replication, the miRNA mutations were built into both the archetype and rearranged BKPyV backgrounds, purified from 293TT cells, and used to infect RPTE cells. We confirmed that the wt viruses recapitulated results seen previously with transfection; i.e., that

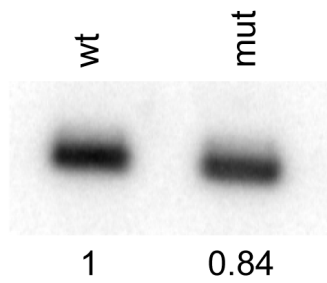


Figure 4.3. The wt Northern blot probe recognizes the mutant miRNA sequence. Equal amounts of wt and miRNA mutant plasmid DNA were digested with *DraI* and run on an agarose gel, blotted, and probed under Northern blotting conditions using the 5P probe. Numbers indicate the quantification of band intensity normalized to the wt. wt, pGEM-Dik; mut, pGEM-Dik miRNA mutant.

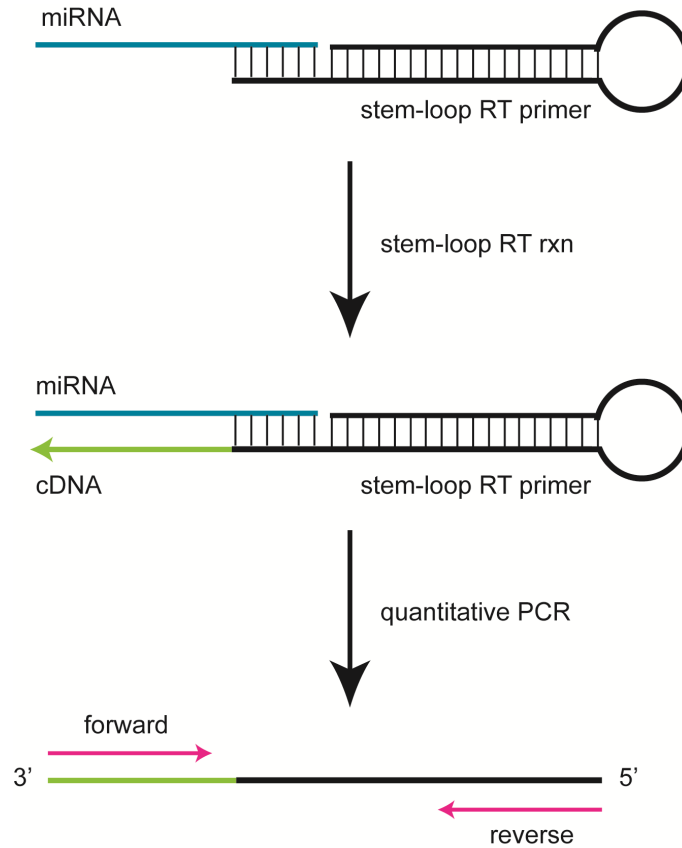


Figure 4.4. Schematic of the miRNA stem-loop qPCR assay. Quantification of mature miRNA by stem-loop RT followed by qPCR. The stem-loop RT primer forms a hairpin on itself and binds to the processed 3' end of the mature miRNA. Reverse transcriptin proceeds to create cDNA. The RT product is then quantified by SYBR qPCR using a forward primer that anneals to the cDNA product and a reverse primer that anneals to the stem-loop RT primer. Figure modified from (Chen et al., 2005; Varkonyi-Gasic et al., 2007).

rearranged variants but not archetype virus are able to replicate in RPTE cells (Broekema et al., 2010). We hypothesized that early mRNA levels would increase in the miRNA mutant archetype virus and this would result in an increase in viral replication. We measured mature 5P miRNA expression by stem-loop qPCR in the mutant virus infection of RPTE cells: miRNA was detected in the wt but not the mutant viruses (Figure 4.5A). Mature miRNA expression is higher in the rearranged variant compared to archetype virus because only the rearranged variant can replicate in RPTE cells. We next examined early transcript and protein production in wt vs. miRNA mutant virus infection at 3 days post infection (dpi) by harvesting total RNA and proteins, respectively. Consistent with the luciferase results, early transcript and protein expression, measured by quantitative reverse transcriptase PCR (qRT-PCR) and Western blotting, respectively, were increased in the archetype miRNA mutant virus infection compared to wt (Figure 4.5B and C). However, mutating the miRNA in the rearranged variant did not increase early transcript production and resulted in only a modest increase in TAg protein expression compared to wt rearranged variant (Figure 4.5B and C), similar to what had been previously reported for mutant SV40 and MPyV. As in 293TT cells, we were unable to detect mature 5P miRNA expression by stem-loop qPCR in the mutant virus infection of RPTE cells. Since early transcript expression was increased by 100 fold in the archetype virus miRNA mutant virus infection, we also measured viral DNA replication by qPCR. Low molecular weight DNA harvested at 3 dpi was normalized to low molecular weight DNA harvested at 1 dpi (input). Viral DNA replication was approximately 50 fold higher in the archetype virus miRNA mutant virus infection compared to wt (Figure 4.5D). We also noted increased VP1 protein expression (Figure 4.5C) in the miRNA mutant virus. This is likely the result of increased replication capacity since it is known that polyomavirus genome amplification results in increased late gene expression

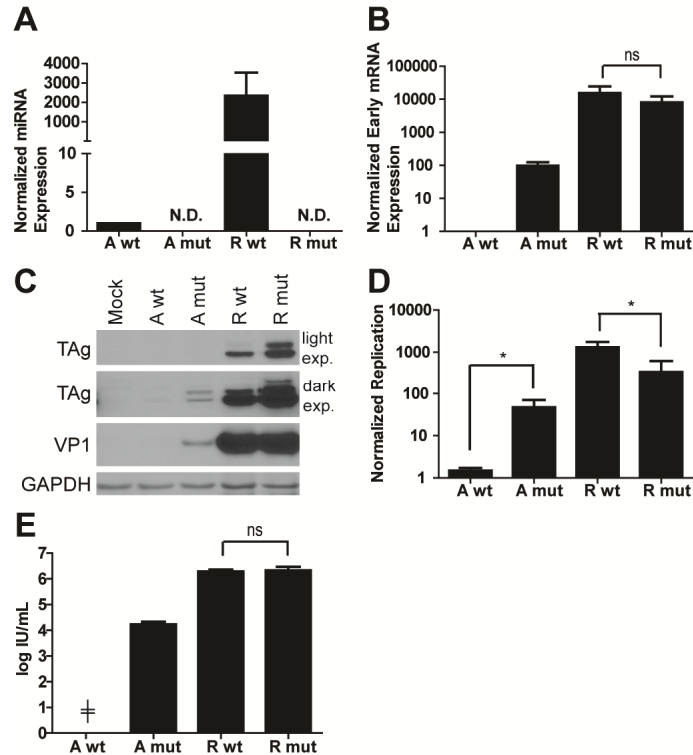


Figure 4.5. The BKPyV miRNA limits viral replication in RPTE cells. RPTE cells were infected with purified wt or miRNA mutant virus at an MOI of 0.01. Total cell RNA was harvested 3 dpi. (A) BKPyV 5P miRNA expression was quantified by stem-loop RT qPCR and normalized to the cellular control hsa-let-7a. (B) Early transcript was measured by qRT-PCR normalized to GAPDH transcript. The A wt sample was arbitrarily set to 1. (C) Total protein was harvested at 3 dpi and was analyzed by Western blotting for the expression of TAg, VP1, and GAPDH. Light and dark exposures of TAg are shown. The blots shown are representative of three independent experiments. (D) Low molecular weight DNA was harvested 1 dpi and 3 dpi and BKPyV DNA was quantified by qPCR. Replicated DNA (3 dpi) was normalized to input DNA (1 dpi). (E) Viral lysates were harvested from cells 3 dpi and progeny were quantified by an IU assay. Each bar is the average from three independent experiments and the error bars are standard deviation. No mature miRNA, early transcript, viral genome or progeny were detected in mock infected samples. N.D., not detected; A, archetype; R, rearranged; wt, wt miRNA; mut, mutant miRNA; ‡, below the limit of detection; *, $p < 0.05$.

(Imperiale et al., 2007a). In contrast, the rearranged variant miRNA mutant did not display increased replication ability, and was actually slightly reduced, compared to wt (Figure 4.5D). Lastly, progeny production was assessed by a VP1 infectious unit (IU) assay at 3 dpi (Broekema et al., 2012). We detected only 1-2 foci/field of view in the wt archetype virus IU assay in each experimental repeat, which is below the effective limit of detection of the assay (8.8×10^3 IU/mL). In contrast, the archetype miRNA mutant virus had a measurable titer of 1.7×10^4 IU/mL (Figure 4.5D). Notably, the rearranged variant miRNA mutant once again behaved similar to other rearranged polyomavirus miRNA mutants, producing the same amount of progeny compared to wt rearranged virus. Increased early mRNA and TAg protein expression shows that the archetype virus miRNA negatively regulates early transcript expression in RPTE cells. Notably, early transcript expression and replication were approximately 100 fold and 50 fold higher, respectively, in the miRNA mutant compared to the wt virus, indicating that the miRNA is largely responsible for limiting archetype virus replication in RPTE cells.

miRNA expression is dictated by the balance of promoter activities. Apart from cellular factors that control mRNA stability, the steady state level of early mRNA is the product of mRNA synthesis, driven by the early promoter, and degradation, driven by the miRNA.

Therefore, we hypothesized that the relative levels of early mRNA and miRNA transcription dictate the difference between archetype virus and rearranged variant replication in RPTE cells. In the archetype virus, early transcript production would be restricted by low early promoter activity combined with high miRNA expression, while the opposite would be true for rearranged variants. The results in Figure 4.5B demonstrate that in the absence of miRNA-mediated degradation, archetype miRNA mutant virus produces approximately 100 fold less early transcript compared to the rearranged variant. This suggests low levels of early promoter activity

in the archetype virus compared to the rearranged variant. Additionally, we hypothesized that the archetype virus would have relatively high levels of late promoter activity driving miRNA expression even early in infection, compared to early promoter activity. This would limit viral replication since the early transcript product TAg is responsible for initiating viral DNA synthesis (Fanning et al., 2009). In contrast, in the rearranged variant, which readily replicates in RPTE cells, we predicted there would be low late promoter activity driving miRNA expression and high early promoter activity driving target early mRNA.

Previously, Gosert et al. developed a bidirectional reporter vector to investigate the functional outcome of BKPyV NCCR rearrangements with respect to early and late gene expression outside the context of infection (Gosert et al., 2008a). In this reporter vector, GFP expression is driven by the late promoter and RFP expression is driven by the early promoter (Figure 4.6A) This study showed that rearranged NCCR structures have high early gene expression and low late gene expression, whereas archetype virus NCCR structure has the opposite pattern, which is consistent with our hypothesis (Gosert et al., 2008a). In this study, rearranged variants were associated with increased cytopathology in patients and increased replication capacity as a result of high early gene expression. We tested our archetype and rearranged NCCRs in this bidirectional reporter assay and found that archetype virus has low early and high late promoter activity, whereas the rearranged variant has high early and low late promoter activity (Figure 4.6B). These data support our hypothesis that archetype virus and rearranged variants have differential promoter activity controlling early and late strand expression.

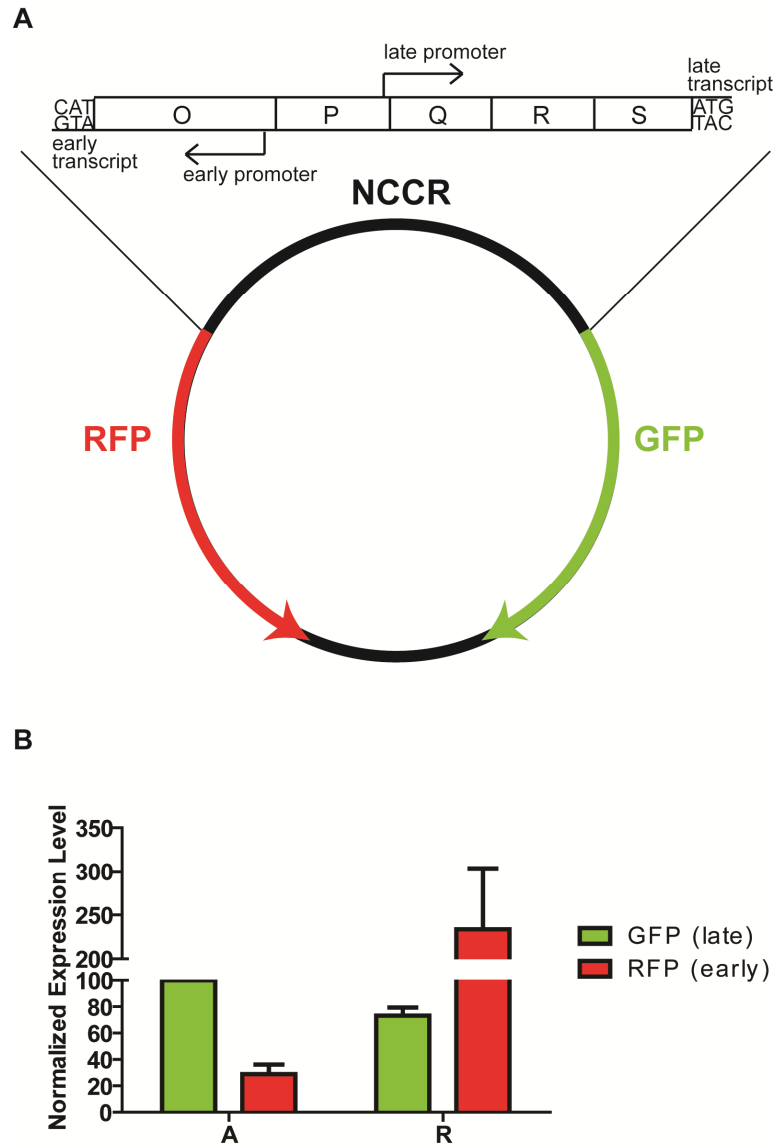


Figure 4.6. Promoter activity of archetype virus vs. rearranged variants. (A) Diagram of bidirectional reporter plasmid. The early promoter drives RFP expression. The late promoter drives GFP expression. (B) 293 cells were transfected with the phRG reporter plasmid containing either the archetype, A, or rearranged, R, NCCR. Expression level is the percentage of GFP (late promoter) or RFP (early promoter) positive cells normalized to GFP positive cells transfected with phRG containing the A NCCR. The archetype expression level was arbitrarily set to 100. A minimum of 100 foci were counted for each fluorescence channel in each independent experiment. Each bar is the average from three independent experiments and the error bars are standard deviation.

To test this hypothesis in a system more relevant to viral infection we created a flipped NCCR structure mutant in both the archetype (A flip) and rearranged (R flip) genomes (Figure 4.7A). In these mutants the late promoter drives early expression and the early promoter drives late expression. Therefore, we were able to measure both early and late promoter activity within the same assay by measuring the late transcript, which is driven by the late promoter in the wt virus and the early promoter in the flip mutant virus. To examine promoter activity during the early phase of infection, without the complication of genome replication, DNA replication was blocked with 40 $\mu\text{g/ml}$ AraC (Jiang et al., 2011) to assess initial miRNA expression in these viruses. RPTE cells were infected with equal genomes of the flip and wt viruses. Total RNA was harvested at 48 hpi, and mature 5P miRNA, the dominant arm, was measured by stem-loop qRT-PCR. Consistent with our hypothesis, the miRNA expression of archetype (A wt) and rearranged (R flip) was high, whereas miRNA expression of R wt and A flip was low (Figure 4.7B). Viral late transcripts were also measured by qRT-PCR, and they paralleled the miRNA expression results (Figure 4.7C). The data from the flip experiment suggest that the miRNA is driven by the late promoter or a unique promoter on the late strand within the NCCR, rather than a unique promoter located outside the NCCR. Additionally, since DNA replication was blocked in this experiment, it is clear that both the miRNA and late transcript can be expressed prior to the onset of DNA replication. Together, these results demonstrate that early in the viral infection, the delicate balance of promoter activity controls the expression of miRNA and its target early mRNA, and this balance dictates the ability to control archetype virus replication in RPTE cells. **miRNA expression is regulated by elements within the NCCR.** The NCCR flip experiments in Figure 4.7 suggest that miRNA expression is regulated or driven by the late promoter or a unique promoter within the NCCR. To distinguish these options we created a mutant virus,

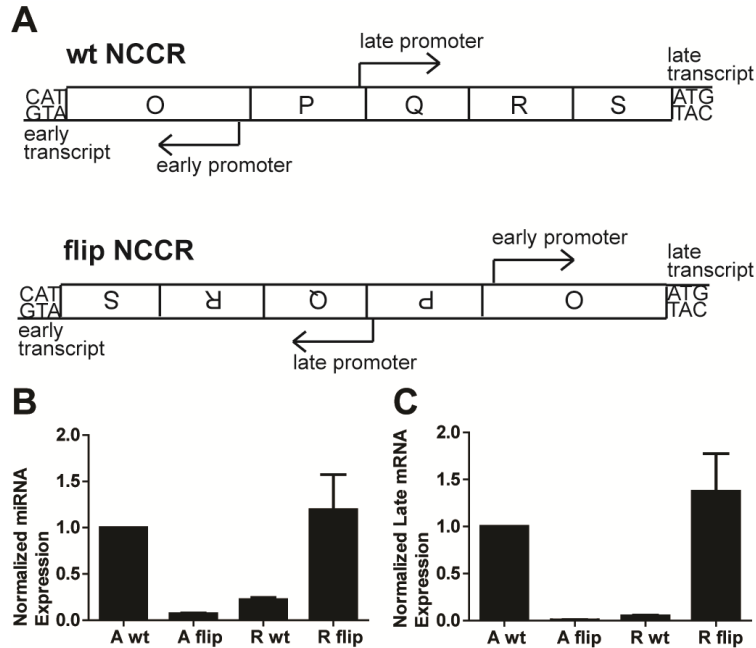


Figure 4.7. Balance of promoter activity in archetype virus vs. rearranged variants. (A) Schematic of the wt and flip NCCR constructs. RPTE cells were infected with 1×10^9 genomes of crude stock virus in the presence of AraC. Total cell RNA was harvested 2 dpi. (B) BKPyV 5P miRNA expression was quantified by stem-loop qPCR and normalized to the cellular control hsa-let-7a. (C) Late transcript was measured by qRT-PCR and normalized to GAPDH transcript. The A wt sample was arbitrarily set to 1. Each bar represents the average from three independent experiments and the error bars are standard error of the mean. No mature miRNA or late transcript was detected in mock infected samples. A, archetype; R, rearranged.

Oblock, lacking the majority of the enhancer region (blocks P, Q, R, and S) and therefore, all identified late transcription start sites. Due to cloning constraints, this mutant contains the first 11 bp of the P block as well as the last 38 bp of the S block. The origin of replication, located in the O block, remains intact in this mutant so that both the Oblock virus and the wt virus are able to replicate in 293TT cells. If both the miRNA and late transcripts are driven by the late promoter, we hypothesized that both would be abolished in the Oblock mutant (Figure 4.8A). The Oblock and wt virus were transfected into 293TT cells and miRNA expression was analyzed by Northern blotting (Figure 4.8B). Mature 5P miRNA was reduced by 5.14 fold (± 1.27) and the mature 3P miRNA was reduced by 7.75 fold (± 2.47) in the Oblock virus compared to the wt virus. We noted a doublet for both the mature 5P and 3P miRNAs, which has been seen for both MPyV and JCPyV, especially late during infection (Seo et al., 2008; Sullivan et al., 2009). Stem-loop qPCR detected the same reduction of mature 5P miRNA expression as Northern blotting, 6.02 fold (± 2.34), in the Oblock mutant (Figure 4.8C). In contrast, late transcript expression was reduced more than two logs compared to wt (Figure 4.8C and D). These results, together with the NCCR flip data, suggest that miRNA expression is at least partially dependent on promoter elements within the NCCR. It is also possible that there is a cryptic promoter on the late strand that can only drive miRNA expression in the Oblock mutant.

Discussion

Archetype BKPyV is thought to be the transmissible form of BKPyV and the form that

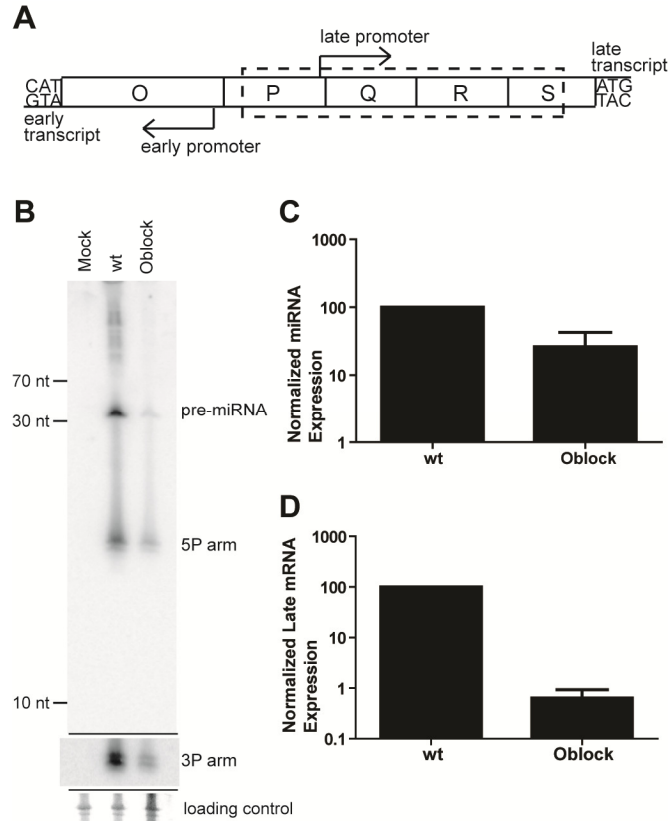


Figure 4.8. miRNA expression is regulated by sequences in the NCCR. 293TT cells were transfected with wt or Oblock mutant virus. Total cell RNA was harvested 5 dpt. (A) Schematic of Oblock mutant virus. Sequence designated by dashed box is deleted. (B) Mature 5P and 3P miRNA were detected by Northern blotting. The blot was hybridized with the 5P probe, stripped, then re-hybridized with the 3P probe. Ethidium bands are shown as a loading control. (C) miRNA expression was quantified by stem-loop qPCR. (D) Late transcript was measured by qRT-PCR normalized to GAPDH transcript. The wt sample was arbitrarily set to 100. Each bar represents the average from three independent experiments and the error bars are standard deviation. No miRNA or late transcript were detected in Mock, mock infected samples. A, archetype; R, rearranged.

establishes persistence in the human host. In this study we set out to investigate the mechanism controlling archetype virus replication in RPTE cells, to provide insights into the mechanism of polyomavirus persistence. We show here that the BKPyV miRNA regulates early mRNA expression, and by extension, viral replication. Significantly, a mutant virus unable to produce mature miRNAs showed a striking increase in early transcript expression, DNA replication, and progeny production, indicating that the miRNA is largely responsible for controlling archetype viral replication. This high degree of regulation is atypical for miRNAs, which are generally considered “fine tuners” of gene expression (Baek et al., 2008). Furthermore, we show that the balance of promoter activity driving expression of high levels of miRNA and low levels of early mRNA uniquely defines archetype virus. We suggest this balance dictates the ability to control archetype virus replication through the action of the miRNA. To our knowledge, this is the first result demonstrating that the polyomavirus miRNA is capable of regulating viral replication.

Polyomavirus miRNAs have many unique attributes. Outside of plants, miRNAs typically form an imperfect duplex with their targets; one arm of the mature miRNA is favored and incorporated into RISC while the other is degraded. However, the polyomavirus miRNAs pair perfectly with early mRNAs that are encoded on the opposite strand, and both the 5P and 3P mature miRNAs are incorporated into RISC for the same target (Seo et al., 2008). Many miRNAs recognize the 3' UTR of a target transcript; however, many polyomaviruses target the transcripts within the 3' coding region. TAg, one of the products of the mRNA that is targeted by the miRNA, plays a major role in the initiation of viral DNA replication at the origin (Fanning et al., 2009). Unexpectedly, mutation or deletion of the SV40 or MPyV miRNA results in increased TAg protein levels but no measurable enhancement of viral replication (Sullivan et al., 2005; Sullivan et al., 2009). As a result, previous studies of the polyomavirus miRNAs, specifically

SV40 and JCPyV, focused on their roles in regulating the host immune response by limiting viral antigen (TA_g) or directly targeting mediators of immune recognition (Bauman et al., 2011; Sullivan et al., 2005).

In this study we define a new role of the polyomaviruses miRNA in controlling archetype viral replication. Previous work with SV40 and MPyV rearranged variants showed no measurable effect on production infectious progeny of mutating the miRNA, which was recapitulated in this study (Figure 4.5E). We also did not detect an increase in replication capacity of the rearranged BKPyV variant miRNA mutant, and in fact saw a small but reproducible decrease (Figure 4.5D). However, we detected a dramatic phenotype in the archetype virus miRNA mutant, resulting in a 100 fold increase in early transcript and a 50 fold increase in replicated viral DNA. This regulation by the miRNA is accomplished through the differential regulation of promoter activity controlling the miRNA and target early mRNA expression in archetype virus compared to rearranged variants. High miRNA expression is effective in limiting archetype virus replication, since this virus also has low early promoter activity driving the target early mRNA (Figure 4.9). The results of the NCCR flip experiment are consistent with the miRNA being expressed from the late promoter. In contrast, the rearranged variants have high early promoter activity driving early mRNA expression and miRNA expression that is low relative to archetype virus. We surmise that the limited amount of miRNA present during rearranged variant infection is unable to impact the level of early mRNA, which is abundant. Indeed, these results are in agreement with our previous work which shows that the archetype virus NCCR is sufficient to confer loss of replication in a rearranged variant background (Broekema et al., 2010). Since all prior work on polyomavirus miRNAs used

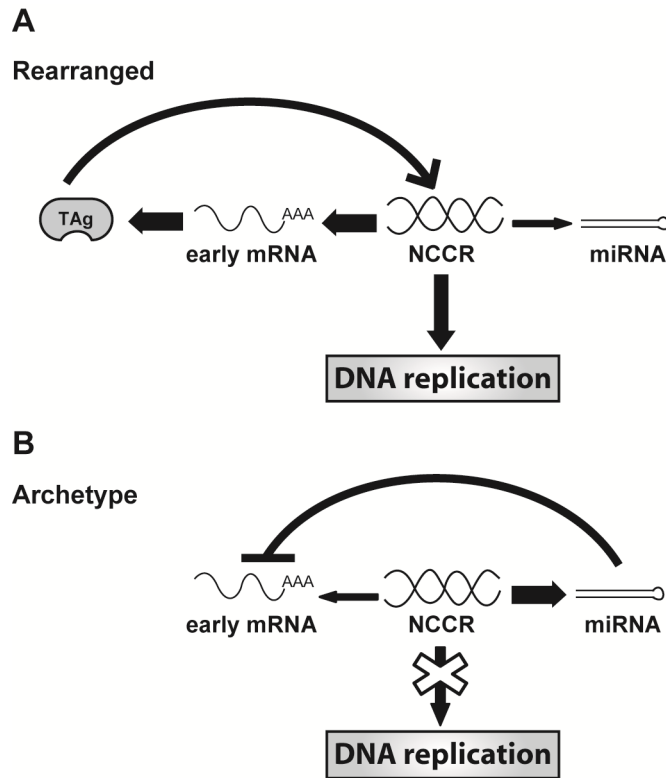


Figure 4.9. Model of miRNA control of archetype virus replication. (A) In the rearranged variants high levels of early mRNA are expressed from high early promoter activity, while the miRNA is only weakly expressed. TAg, translated from early mRNA, binds to the origin of replication and drives DNA replication. (B) Archetype virus early mRNA is weakly expressed from the early promoter while miRNA is robustly expressed and targets early mRNA for degradation. Therefore DNA replication is blocked in archetype virus in RPTE cells.

rearranged variants, this explains why no previous studies have observed a functional role for polyomavirus miRNA in regulating viral replication. Our results with rearranged BKPyV also support this conclusion.

We originally hypothesized that the miRNA is expressed from the viral late promoter as it is expressed from the late strand. The late promoter is nebulous and has no clearly defined elements. This is likely a mechanism to cope with the large number of natural NCCR rearrangements that occur within this region (Moens et al., 1995). As a result, late transcription has to be able to initiate at a variety of sites depending on which sequence blocks are present and in what order, and take into account the distance to the start codon. It is possible that it has a unique promoter within the NCCR or that something in the NCCR is able to uniquely regulate miRNA expression. Our results from the Oblock experiment indicate that the miRNA may be able to use a promoter other than the viral late promoter. The early promoter TATA box on the opposite strand is also a potential TATA box on the late strand and could theoretically function for the miRNA. Alternatively, the miRNA may be able to use more than one promoter for its expression. Therefore when the late promoter is deleted as in the Oblock mutant, the virus may be able to use another, as yet unidentified promoter. Another alternative is that the late promoter is composed of multiple segments, one of which may be more active early in infection while the other is more active later. Specifically the early-active segment could potentially drive miRNA expression whereas the late-active segment could drive late transcript expression but may have limited activity early. The adenovirus major late promoter is arranged in this manner with an early active segment and a segment that has both early and enhanced late activity (Logan et al., 1986). Fine mapping of the miRNA promoter is an interesting area for future study.

Many viral miRNAs were discovered in cells latently infected with herpesviruses, and as a result, viral miRNAs have been suggested to play an important role in establishing or maintaining viral latency or persistence (Grundhoff et al., 2011). Moreover, several Kaposi sarcoma herpesvirus miRNAs are vital for directly and indirectly controlling lytic transcript expression (Lei et al., 2010; Lu et al., 2010a; Lu et al., 2010b). While previous studies have described the polyomavirus miRNAs as being expressed late during infection (Seo et al., 2008; Sullivan et al., 2005), our stem-loop qPCR data show that the miRNA is expressed before the onset of DNA replication. At later time points, the miRNA can be detected by Northern blotting, a less sensitive method, indicating that the miRNA accumulates as the virus replicates. Thus, the archetype virus late promoter is active early, prior to DNA replication. We also noted in the Northern blot that there was an accumulation of high molecular weight unprocessed miRNA precursors and a decrease in the pre-miRNA band in the miRNA mutant virus, indicating that the defect in miRNA biogenesis likely occurs prior to pre-miRNA processing (Kincaid et al., 2012a). We show here that the balance of promoter activity controlling miRNA and its target early mRNA expression regulates archetype BKPyV replication. Since archetype virus is the form of BKPyV that is believed to be transmitted and establish persistence in the host, the regulation of replication by the miRNA is likely essential to the ability of this form of the virus to persist in the host. We suggest that the polyomavirus miRNA plays a major role in the establishment of viral persistence since we noted a dramatic reduction of regulation in archetype virus replication in the miRNA mutant virus. Polyomaviruses have miRNAs antisense to their early coding regions, and therefore regulation of viral replication is likely a common mechanism employed by polyomaviruses to establish a life-long persistent infection (Grundhoff et al., 2011). In summary, these results show that archetype virus replication is uniquely controlled in RPTE cells by the

balance of promoter activity driving high miRNA expression relative to low early mRNA expression. This has implications for the increasing number of immunocompromised patients affected by polyomavirus-associated diseases.

Materials and Methods

Cell culture. Primary human RPTE cells (Lonza) were maintained in renal epithelial growth medium for up to six passages (Abend et al., 2007). 293TT cells were obtained from Chris Buck at the National Cancer Institute and were maintained under 400 µg/ml hygromycin selection (Buck et al., 2004). 293 (Graham et al., 1977) and 293TT cells were maintained in Dulbecco's modified Eagle medium with 10% fetal bovine serum and 100 U/ml penicillin, 100 µg/ml streptomycin. All cells were grown in a 5% CO₂ environment in a humidified 37°C incubator.

miRNA folding prediction. The mfold RNA folding form was used to predict the BKPyV miRNA wt and mutant RNA structures (<http://mfold.rna.albany.edu/>) (Zuker, 2003).

DNA constructs, transfection, infection, and luciferase assays. Archetype virus and rearranged variant miRNA mutated viruses were created using site-directed mutagenesis performed using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene) as described previously (Abend et al., 2008) on both pGEM-Dik and –Dunlop (Broekema et al., 2012) to create the miRNA mutant in the archetype and rearranged background, respectively. Three point mutations were created in the pre-miRNA sequence G13A, U43G, and U46G (where 1 is the 5' start of the pre-miRNA) using primers A, B, and C and their reverse complements (Table 4.1). Mutations and the integrity of the early and late coding regions were confirmed by DNA sequencing. The wt and mutant viral genomes were transfected into 293TT cells to produce crude lysates (Broekema et al., 2012). Viral stocks were expanded in 293TT cells and purified by

Table 4.1. List of primers used for cloning, site-directed mutagenesis, and real-time PCR.

Primer	Sequence (5'-3')
Primers used for cloning and site-directed mutagenesis ^a	
A G13Amut	GCTGAAGTATCTGAGACTTGAGAAGAGCATTGTGATTGGG
B U43Gmut	GGGATTCAGTGCTGGATCCATGTCCAGAG
C U46Gmut	GGGATTCAGTGCTGGAGCCATGTCCAGAG
D TAg miRNA FOR	GAAGGCACCATGGGGAAATGTATTCTTGAT
E TAg miRNA REV	GAAGGTGCCTGACCTTTGGGAATCTTCAGC
F TAg concat FOR	ATGCTCGAGCGGCCGCCAGTGTGATGGATA
G TAg concat REV	GCATCTAGAGTAACGGCCGCCAGTGTGCTG
H OflipeLate	ATTATACCGCGGGATTTCCCAAATAGTTTTGC
I SflipeEarly	ATGGTAACTAGTCTCGCAAACATGTCTGTC
J phRG NCCR MluI	AAAAACGCGTCATTTTTGCAAAAATTGCAAAAAGAATAGG
K phRG NCCR BssHII	TTTTGCGCGCCTGGCGCAGAACCATGGCCTT
L SpeI Oblock FOR	GGA ACTAGT GATTTCCCAAATAGTT
M SacII Oblock REV	TA ACCGCG TCCTCCCTGTGGCCTTTT
Primers used for real-time PCR ^b	
N 5P miRNA	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGA TACGAC ATGCTC
O 5P neg control	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGA TACGAC CCAAGT
P miRNA qPCR FOR	GCCTCGATCTGAGACTTGGGAA
Q Stemloop qPCR REV	GTGCAGGGTCCGAGGT
R Early FOR	AAGGAAAGGCTGGATTCTGA
S Early REV	TGTGATTGGGATTCAGTGCT
T Late FOR	ACAGGCCACAAATATCAGCA
U Late REV	TGTGACCAACACAGCTACCA
V GAPDH FOR	GCCTCAAGATCATCAGCAAT
W GAPDH REV	CTGTGGTCATGAGTCCTTCC

^a Restriction enzyme sites are underlined and point mutations are shown in bold in the primer sequence.

^b Underlined portion corresponds to the part of the primer that binds to the 3' end or the middle (negative control) of the 5P miRNA.

density centrifugation on CsCl gradients (Jiang et al., 2009b). Viral titers were determined by VP1 IU assay (Broekema et al., 2012). We set the limit of detection for the IU assay as a minimum of 20 foci/field of view.

Constructs pcDNA3.1puro, pcDNA3.1dsRluc, pcDNA3.1dsFFluc, and pcDNA3.1BKV miRNA were kindly provided by C. Sullivan and G. Seo (University of Texas-Austin) (Seo et al., 2008). The region of the BKPyV genome corresponding to the miRNA target sequence in the early mRNA was PCR amplified and subcloned into the 3' UTR of pcDNA3.1dsRluc as previously described, as a single target as opposed to a concatemered target, using primers D, E, F, and G (Table 4.1). Site-directed mutagenesis was used as above to create the mutated BKPyV miRNA expression vector pcDNA3.1BKVmiRNAmutant.

293 cells were plated into 12-well dishes and transfected with Turbofect (Fermentas). Cells were transfected with 100 ng pcDNA3.1dsRlucBKV TAg, 50 ng pcDNA3.1dsFfluc (transfection control) and 1850 ng pcDNA3.1BKVmiRNA or pcDNA3.1BKVmiRNAmutant expression vector for a total of 2 µg DNA. Cell lysates were harvested 48 hours post transfection (hpt) and analyzed using the Dual-luciferase reporter assay system according to the manufacturer's instructions (Promega) and read on a Promega GloMax 96 Microplate luminometer.

The archetype virus and rearranged variant NCCR were cloned into the phRG promoter construct as previously described (Gosert et al., 2008a), kindly provided by Hans H. Hirsch (Universität Basel). Constructs were confirmed by DNA sequencing. 293 cells were transfected with TransIT LT-1 transfection reagent (Mirus Bio, Madison, WI) according to the manufacturer's instructions and analyzed at 2 dpt (Gosert et al., 2008b).

NCCR flip constructs were created in the Dik-3-site (archetype virus) and Dunlop-3-site backbones (rearranged variant), which replicates equivalently to wt Dunlop in RPTE cells (Broekema et al., 2010). . These vectors contain *SpeI* and *SacII* sites in the O and S blocks of the NCCR, respectively. The NCCR structure was flipped by adding a *SacII* to the O block using primer H and a *SpeI* site to the S block using primer I (Table 4.1). This resulted in a flipped NCCR structure where the top strand becomes the bottom strand and vice versa. PCR products were gel purified and ligated into digested Dik and Dun-3-site backbones using T4 DNA ligase. The flip constructs were confirmed by DNA sequencing. Religated genomes were transfected into 293TT cells and crude lysates were harvested 7 days post transfection (dpt). Lysates were freeze/thawed 3x and genomes were quantified by real-time PCR (Broekema et al., 2012).

For infection, cells were pre-chilled for 15 min at 4°C. Cells were exposed to virus diluted into media (DMEM for 293TT cells or REGM for RPTE cells) at an MOI of 0.01 or 1×10^9 genomes/cell (NCCR flip experiments) and incubated for 1 h at 4°C. The viral inoculum was then removed and infection was initiated by adding warm media and transferring the cells to 37°C.

The Oblock mutant virus was created in the Dunlop-3-site backbone (Broekema et al., 2010). The truncated NCCR was created by PCR amplifying the O block with the *SpeI* site using primer L and adding a *SacII* site into the beginning of the P block using primer M (Table 4.1). The PCR product was gel purified and ligated into the Dun-3-site backbone using T4 DNA ligase. The construct was confirmed by DNA sequencing. 293TT cells were transfected with Lipofectamine 2000 transfection reagent (Life Technologies) according to the manufacturer's instructions and total RNA was harvested 5 dpt.

Northern, Western, and Southern blotting. Total RNA was harvested from cells with Trizol (Life Technologies) and processed using the Direct-zol RNA MiniPrep (Zymo Research). RNA was run on a Tris-borate-EDTA-urea-15% polyacrylamide gel and transferred to Hybond H+ membrane as previously described (McClure et al., 2011). The blot was probed with Starfire probes according to the manufacturer's instructions (IDT). The following probes were used: BKV 5P probe 5'-CAATCACAATGCTCTTCCCAAGTCTCAGATACTTCA-3', BKV 3P probe 5'-ACTGAAGACTCTGGACATGGATCAAGCACTGAATCC-3'.

E1A lysis buffer was used to harvest total cell proteins, which were quantified and immunoblotted as previously described (Broekema et al., 2012; Jiang et al., 2009b). The following antibodies and concentrations were used: pAb416 (Harlow et al., 1981) for TAG at 1:3000; P5G6 for VP1 at 1:10,000; and Ab9484 (Abcam) for GAPDH at 1:10,000.

Southern blotting was performed as described previously described (Broekema et al., 2010). Plasmid DNA, pGEM-Dik wt and miRNA mutant, were digested with *DraI*. The blots were probed under Northern blotting conditions as previously described (McClure et al., 2011). The Southern blot was quantified using a Typhoon phosphorimager and Imagequant software (GE).

Quantitative PCR. The miRNA stem-loop real-time PCR protocol for the BKPyV 5P miRNA, kindly provided by Chris Sullivan, was adapted from previously published protocols (Chen et al., 2005; Varkonyi-Gasic et al., 2007) All RNA was harvested at the indicated time points as described in the Materials and Methods. RNA integrity was confirmed by electrophoresis on an agarose gel. To quantify the BKPyV 5P miRNA, a stem-loop reaction (Varkonyi-Gasic et al., 2007) was performed with 1 µg of total RNA, 0.5 µl 10 mM dNTP mix, 1 µl of 1uM stem-loop RT primer N or the negative control primer recognizing the middle of the mature miRNA primer

O (Table 4.1). The stem-loop primer annealing program is 5 min at 65°C and 2 min on ice. The stem-loop product is then added to an RT reaction using SuperScript III Reverse Transcriptase (Invitrogen) containing 4 µl First-strand buffer, 2 µl 0.1 M DTT, 0.1 µl SUPERase (Ambion), and 0.25 µl (50 units) Superscript III reverse transcriptase. The pulsed RT program was performed with the following parameters: 30 min at 16°C, followed by 60 cycles of 30 s at 30°C, 30 s at 42°C, and 1 s at 50°C, then 5 min at 85°C. Next, real-time PCR was performed with the RT product in a total volume of 20 µl using 10 µl Power SYBR green PCR master mix (Applied Biosystems), 2 µl RT product, 1 µl 10 µM primer P and Q (Table 4.1), and 6 µl H₂O. Amplification was performed in 96-well PCR plates using the iCycler iQ5 real-time detection system (Bio-Rad) with the following program: 5 min at 95°C, 35 cycles of 95°C for 5 s, 60°C for 10 s, and 72°C for 1 s. Analysis of serial dilutions of RNA from infected RPTE cells show that from 1 ng to 1 µg, a range which includes the conditions used in our experiments, the BKPyV 5P stem-loop assay is linear ($R^2 = 0.991$) and that from 0.5 ng to 500 ng, the hsa-let-7a stem-loop assay is linear ($R^2 = 0.998$). Results are presented as fold change in miRNA transcript levels relative to levels in wt archetype virus-infected samples, normalized using $2^{-\Delta\Delta C(t)}$ method (Livak et al., 2001) to a human miRNA control (hsa-let-7a). miRNA copy number was determined by comparison to a standard curve using RNA oligos (IDT) corresponding to the BKPyV 5P or hsa-let-7a 5P miRNAs.

To quantify viral early and late transcripts, total RNA was harvested and treated with DNase (Promega) to eliminate contaminating DNA. To generate cDNA, a reverse transcription reaction was performed with 50 ng of RNA template using SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. Real-time PCR was performed in a total volume of 25 µl using 12.5 µl Power SYBR green PCR master mix (Applied Biosystems),

2.5 µl cDNA template, and 300 nM each primer: for early transcript, primers R and S; for late transcript, primers T and U; and for GAPDH, primers V and W (Table 4.1). Amplification was performed in 96-well PCR plates using the iCycler iQ5 real-time detection system (Bio-Rad) with the following program: 2 min at 50°C, 10 min at 95°C, 40 cycles of denaturation at 95°C for 15 s, and annealing and extension at 58°C (for early) or 57°C (for late) for 30 s. Results are presented as fold change in transcript levels relative to levels in wt archetype virus-infected samples, normalized to GAPDH using the $2^{-\Delta\Delta C(t)}$ method (Livak et al., 2001) and the A wt sample is arbitrarily set to 1.

To quantify virus DNA replication by qPCR, low molecular weight DNA was isolated using a modified Hirt isolation protocol, real-time PCR was performed and the data were analyzed as previously described (Hirt, 1967; Jiang et al., 2011). Viral DNA replicated at 3dpi was normalized to input DNA harvested 1 dpi using a ΔC_t method.

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Chapter V

Discussion

BK polyomavirus (BKPyV) is a ubiquitous virus that establishes a persistent infection in the human host urinary tract. Initial infection occurs in early childhood and is thought to be asymptomatic. BKPyV is not known to cause disease in healthy individuals but periodically can be asymptotically shed in the urine (Knowles, 2001). The virus may reactivate under immunosuppressed conditions to cause polyomavirus-associated nephropathy (PVAN) or hemorrhagic cystitis (HC) in renal and bone marrow transplant (BMT) recipients, respectively. BKPyV has a small double-stranded circular DNA genome that is divided into three major genetic regions: the early coding region, the late coding region, and the non-coding control region (NCCR). Additionally, there are two forms of BKPyV, which are differentiated by their NCCR structure, termed archetype virus and rearranged variants. Archetype virus is the transmissible form of BKPyV and is characterized by a linear O-P-Q-R-S block structure of its NCCR, while rearranged variants have duplications and/or deletions in the sequence blocks and often arise during disease progression. Rearranged variants grow robustly in the cell culture model of lytic replication, primary renal proximal tubule epithelial (RPTE) cells, whereas archetype virus do not produce progeny virus in this cell type.

Previous work in our laboratory and others has focused on the role of the host immune system in regulating viral replication. Roles for innate and adaptive immunity have been

described to limit replication of rearranged variant, the only form culturable. It is likely that any or all of these immune mechanisms contribute to limiting archetype virus lytic reactivation in the host, thereby promoting a state of viral persistence. This dissertation describes a cell culture model to propagate archetype virus and investigates the viral mechanisms able to regulate archetype virus *in vitro* replication. This research has implications contributing to understanding the mechanism of viral persistence in the human host, which will be discussed in a overview model of viral persistence.

Chapter summary

In Chapter II we investigated which major genetic region determines replication ability of archetype virus vs. rearranged variant in RPTE cells. We developed a swap vector system in which each of the three major genetic regions of BKPyV were exchanged between archetype virus and a rearranged variant to create all possible combinations. Replication ability was assessed for each viral chimera in RPTE cells. The rearranged variant NCCR was sufficient to confer replication ability in an archetype virus background. The NCCR does not encode any viral proteins but does contain divergent promoters and transcription factor binding sites for both the early and late coding regions. Also, there are *cis*-acting sequences near the origin that affect chromatin structure and therefore DNA replication (de Villiers et al., 1984; Muller et al., 2000). Therefore the regulatory functions of the NCCR differentiate the replication phenotype of the two forms of BKPyV in RPTE cells. Additionally, we analyzed several BKPyV clones isolated from the urine of an HIV patient using the swap vector system and showed that as rearrangement complexity of the NCCR increases, so do replication ability and progeny production.

In Chapter III, we assessed the ability of TAg overexpression to rescue archetype virus replication and resultantly developed a cell culture model to propagate archetype BKPyV.

Previously, in Chapter II, we observed that archetype virus does not produce a detectable level of TAg protein when the viral genome is transfected into RPTE cells. Concordantly, previous research showed that archetype virus has a low level of early promoter activity compared to rearranged variants (Gosert et al., 2008; Markowitz et al., 1990). Since TAg is required to initiate DNA replication, we hypothesized that adding TAg in *trans* would rescue archetype virus replication. Indeed, SV40 TAg expression in COS-7 cells efficiently replicates archetype JC polyomavirus (JCPyV) DNA (Hara et al., 1998). Recently, Buck et al. developed 293TT cells, which express high levels of SV40 TAg, for the purpose of producing HPV pseudovirions (Buck et al., 2005). In this system HPV capsid protein expression plasmids are co-transfected with a pseudogenome containing an SV40 origin, which can be replicated by the TAg in 293TT cells and packaged within the pseudovirions. SV40 TAg is 85.9% similar to BKPyV TAg and is also able to initiate replication at the BKPyV origin of replication (Daniel et al., 1996; Mahon et al., 2009; Sock et al., 1993). Therefore, we tested the ability of archetype virus to replicate in 293TT cells. We found that archetype virus replicated and produced infectious progeny in 293TT cells (Broekema et al., 2012). Additionally, 293TT cells support progeny production of JCPyV but not Merkel cell, KI, or WU polyomavirus. The coding sequences of MCPyV, KIPyV, and WUPyV TAg have a 47.6%, 64.4%, and 62.8% similarity to SV40 TAg, respectively, whereas BKPyV has 85.9% and JCPyV has 84.1% similarity. Therefore, the inability of these polyomaviruses to replicate in 293TT cells is likely due to the fact that SV40 TAg is not similar enough to bind and initiate replication from the origins of replication of MCPyV, KIPyV, and WUPyV. This also suggests that cell culture systems overexpressing the TAg of a specific polyomavirus may rescue replication of as yet unculturable corresponding polyomaviruses.

Previous attempts to passage archetype virus in cell culture resulted in NCCR rearrangement in as little as one passage (Rubinstein et al., 1991). However, in 293TT cells the archetype NCCR structure remains intact, suggesting that there is little or no selective pressure to produce rearrangements in this cell type. These results are significant because this is the first system in which archetype virus can be propagated in the laboratory and used for further study.

In Chapter IV, we investigated the mechanism regulating viral replication in RPTE cells. As discussed in Chapters II and III, archetype virus, which is the transmissible form of BKPyV that establishes a persistent infection in the human host, is unable to replicate in RPTE cells. We tested the hypothesis that the BKPyV miRNA, which had previously been shown to regulate early mRNA (Seo et al., 2008), could regulate archetype virus replication in RPTE cells. We found that this regulation was unique to the archetype virus and was not seen in the rearranged variant, which corresponds to what has been seen for previous rearranged polyomaviruses (Sullivan et al., 2005; Sullivan et al., 2009). This unique regulation is the result of differential regulatory elements within the NCCR in archetype vs. rearranged variants. Archetype virus has low early promoter activity, which controls expression of the miRNA target early mRNA. High miRNA expression early during infection may be controlled by the late promoter or a unique promoter within the NCCR. Alternatively, rearranged variants have reversed expression levels of miRNA and its target mRNA, i.e. low levels of miRNA and high level of target mRNA. These data have implications for the mechanism of archetype virus persistence. In Chapter IV we also provide evidence contrary to the canonical temporal expression pattern described for polyomaviruses of early gene expression, then DNA replication, and late gene expression dependent on replication. Specifically, we showed that archetype virus does not replicate in RPTE cells and late gene expression is higher compared to early gene expression. In agreement,

others have shown that the late promoter is always firing, even in the absence of both TAg and viral DNA replication (Hyde-DeRuyscher et al., 1988; Keller et al., 1984).

Additionally, we have ongoing work toward establishing a model of archetype virus persistence in urothelial cells. Urothelial cells were chosen because one major diagnostic tool for BKPyV infection is the presence of decoy cells in patient urine (Ahsan et al., 2006). Decoy cells are urothelial cells with a large basophilic nuclear inclusion. Additionally, BKPyV TAg has been detected by immunohistochemistry in the stratified urothelium of patient samples (Roberts et al., 2008). We hypothesize that undifferentiated urothelial cells support episomal maintenance of the archetype BKPyV viral genome and that cellular differentiation may support viral replication akin to HPV persistence in keratinocytes (Moody et al., 2010). However, the BKPyV mechanism of persistence and reactivation may be different from HPV, in that BKPyV encodes a regulatory miRNA, similar to herpesviruses whose miRNAs have been shown to be involved in promoting viral latency (Murphy et al., 2008), whereas HPV does not. Therefore, BKPyV may combine features of both HPV persistence and herpesvirus latency. In the appendix we show preliminary data that archetype virus DNA is specifically maintained in urothelial cells over an extended timecourse and that no detectable progeny are produced from these cells. These preliminary results lay the groundwork for investigating the mechanism of archetype BKPyV persistence and what role, if any, the viral miRNA plays in this process.

To confirm that urothelial cells are indeed a model of archetype virus persistence, it is necessary to show that the maintained DNA remains infectious, as shown by defining a stimulus of reactivation. We hypothesize that we can artificially stimulate reactivation of persistent BKPyV in urothelial cells. Under immunosuppressed and cellular stress states, such as conditions seen in transplant patients, viruses may switch from latent to lytic infection.

Differentiation, hypoxia, immunosuppressants, and cellular stress-inducing conditions will be tested to activate persistent genomes to a lytic state. One possible candidate immunosuppressant drug is dexamethasone, a steroid. This drug has been shown to reactivate latent herpesviruses (Halford et al., 1996; Winkler et al., 2000). During kidney transplantation, the organ being transplanted is deprived of oxygen during transport, defined as hypoxia. Hypoxia can also reactivate latent herpesviruses (Davis et al., 2001) and is therefore a good candidate stressor to test because it may be a natural stressor for PVAN patients. Additionally, cellular stresses such as heat stress, reactive oxygen species, and endoplasmic reticulum stress reactivate latent viruses (Halford et al., 1996; Li et al., 2011; Shirley et al., 2011). Another potential (albeit artificial) method of inducing archetype virus reactivation is by overexpressing TAg, which we showed increases archetype virus DNA replication (Broekema et al., 2012). As discussed in Chapter IV, archetype BKPyV has low early promoter activity, and therefore early mRNA expression, which contributes to the ability of the miRNA to control archetype virus replication. One possible method of supplying TAg to persistent archetype virus is by superinfecting with a rearranged variant. We can then measure any potential increase in archetype virus DNA by qPCR using primers specific for the archetype NCCR.

One potential difficulty in identifying a method to induce BKPyV viral reactivation in cell culture is that a combination of reactivation stimuli may be required. In the case of urothelial cells, it is possible that cellular differentiation may be required to induce reactivation, as is the case for HPV (del Mar Pena et al., 2001), or differentiation in combination with cellular stresses may be required. RPTE cells, a lytic model of rearranged variant replication, are differentiated, which may contribute to replication of these variants. However, another possible requirement for reactivation may be cell cycle exit, which is the case for mouse polyomavirus (MPyV) (Atencio

et al., 1994). The newborn mouse kidney, which is actively differentiating, is permissive for MPyV infection and persistence, while the adult kidney, which is mature and quiescent, is non-permissive. Cell cycle exit occurs when cells undergo terminal differentiation, such as the upper cell layers of keratinocytes and the urothelium. Currently, we are interested in establishing an *in vitro* model of stratified, differentiated, and polarized urothelial cells in a transwell system in our laboratory to test whether terminal differentiation alone or in combination with cellular stresses can reactivate persistent archetype viral DNA.

Urothelial cells form a true stratified epithelium consisting of basal, intermediate, and superficial cells, similar to keratinocytes (Jost et al., 1989). We think that archetype BKPyV maintenance in urothelial cells may be akin to HPV maintenance in keratinocytes (Moody et al., 2010). Specifically we hypothesize that BKPyV DNA is maintained episomally in the basal cells and replication is induced upon terminal cellular differentiation in stratified cultures. Viral progeny are then assembled in the upper granular layer and released from the superficial layer of the stratified cell culture model. Both urothelial cells and keratinocytes are polarized and display increasing differentiation complexity from basal to surface cells. Urothelial cells, which are maintained in defined, low serum and calcium media, differentiate and polarize in the presence of high serum and calcium and plating on transwell inserts (Southgate et al., 1994).

Another factor supporting the model that BKPyV, like HPV, naturally reactivates as a result of cell differentiation is that there have been many recently discovered polyomaviruses shed from the skin, including Merkel cell polyomavirus, Trichodysplasia spinulosa-associated polyomavirus, and human polyomaviruses-6, -7, -9, and -10. Interestingly, there is evidence that the superficial layers may be more transcriptionally active than the basal layers, based on fine granular appearance of the nuclei compared to condensed nuclei (Jost et al., 1989). This is an

interesting area of future research as a possible mechanism of archetype virus propagation that may require cell type specific transcription factors. For example, the archetype virus early promoter may be activated in the superficial urothelium to a greater degree than the promoter driving miRNA expression, which we showed in Chapter IV uniquely inhibits archetype virus replication. This would result in a high level of early mRNA that the miRNA would be unable to inhibit, shifting the balance toward archetype virus replication. Investigation of a model of archetype BKPyV persistence is important because a natural host cell has not been described for any archetype human polyomavirus, and confirmation would therefore eliminate a major gap in knowledge in the field. Overall, this work contributes towards our model of viral persistence described in Chapter V (Figure 5.2).

In addition to continuing studies on BKPyV persistence, the work described in this dissertation allows for future studies of archetype BKPyV. Specifically, we propose two areas of research described below.

Future areas of study for archetype BKPyV

- 1. Identifying relevant cellular targets of the BKPyV miRNA**
- 2. The differences between the archetype virus and rearranged variant particle**

Identifying relevant cellular targets of the BKPyV miRNA

In Chapter IV, we showed that the BKPyV miRNA is responsible for uniquely regulating archetype virus replication due to differential NCCR regulatory elements, leading to high miRNA expression relative to low levels of its target early mRNA expression. Additionally, we showed preliminary data mapping these miRNA regulatory sequences to within the NCCR.

However, we are also interested in identifying cellular targets of the BKPyV miRNA, especially targets that may influence viral replication. It is known that miRNAs may have hundreds of target mRNAs, since it is common to recognize a target with imperfect complementarity (Baek et al., 2008). Therefore, in addition to targeting the viral early mRNA, the BKPyV miRNA possibly has many relevant cellular targets. Indeed, one identified cellular target of the BKPyV and JCPyV identical 3-prime (3P) arm is reported to be the stress-induced ligand ULBP3, which is recognized by NKG2D, a receptor for natural killer (NK) cells (Bauman et al., 2011).

To initiate our investigation of host cellular targets we used the computational miRNA target prediction software DIANA microT v3.0 (<http://diana.cslab.ece.ntua.gr/>) to identify possible cellular targets (Maragkakis et al., 2009a; Maragkakis et al., 2009b). The 5-prime (5P) miRNA was predicted to target 203 target sites in 54 genes (Table 5.1) and the 3P miRNA was predicted to target 100 target sites in 30 genes (Table 5.2). Notably, there were eight shared targets between the 5P and 3P arm miRNAs, which may indicate a greater likelihood that these are real miRNA targets. Therefore, there are 76 potential cellular gene targets as candidates for future study.

Several candidate genes stand out based on their function and the role they may play in viral infection. Many of the identified genes, including ONECUT2, KLF2, PIP4K2B, CRT3, MLL, MXD1, ZNF629, BACH2, ZNF468, ZNF490, and ZNF283 have roles in transcription regulation, which could directly affect viral gene expression. One predicted gene target, *FREM2*, plays a role in maintaining the differentiated state of renal epithelia (Pavlakakis et al., 2011). Since BKPyV infects renal epithelial cells, *FREM2* is a promising candidate for further investigation. Another target, *THSD4*, also known as *ADAMTSL6*, may attenuate the signaling of TGF- β , possibly creating sequestered pools TGF- β (Saitoh et al., 2009). TGF- β regulates the BKPyV

Table 5.1. BK polyomavirus 5P miRNA [bkv-miR-B1-5p] predicted targets.

Gene name ^a	Ensembl Gene ID	miTG score	Precision	SNR	Biological Process
GRIN2A	ENSG00000183454	14.76	0.12	1.12	Ion Transport
ONECUT2	ENSG00000119547	14	0.12	1.12	Transcription regulation
KLF12	ENSG00000118922	13	0.12	1.12	Transcription regulation
ATP11A	ENSG00000068650	13	0.12	1.12	Ion Transport
LRRC27	ENSG00000148814	12	0.12	1.12	
CSNK1G1	ENSG00000169118	12	0.12	1.12	Wnt signaling pathway
FAM123A	ENSG00000165566 11.000.120.56	11	0.12	0.56	Wnt signaling pathway
C1orf21	ENSG00000116667	11	0.12	0.56	
PXMP4	ENSG00000101417	11	0.12	0.56	
RIPK5	ENSG00000133059	11	0.12	0.56	
TTN	ENSG00000155657	11	0.12	0.56	mitotic chromosome condensation, regulation of protein kinase activity
DLGAP2	ENSG00000198010	10	0.12	0.52	synaptic transmission
KIAA1244	ENSG00000112379	10	0.12	0.52	negative regulation of phosphatase activity
NTRK3	ENSG00000140538	10	0.12	0.52	differentiation
DST	ENSG00000151914	10	0.12	0.52	cell adhesion
MDN1	ENSG00000112159	10	0.12	0.52	protein complex assembly
SLC5A3	ENSG00000198743	9	0.12	0.61	Ion Transport
TMEM123	ENSG00000152558	9	0.12	0.61	oncosis
TMEM154	ENSG00000170006	9	0.12	0.61	
FREM2	ENSG00000150893	9	0.12	0.61	cell adhesion
SLC44A1	ENSG00000070214	9	0.12	0.61	transport
PDPK1	ENSG00000140992	9	0.12	0.61	transcription regulation
PIP4K2B	ENSG00000141720	9	0.12	0.61	cell surface receptor signaling pathway
PFKFB2	ENSG00000123836	9	0.12	0.61	glycolysis
SORCS2	ENSG00000184985	9	0.12	0.61	
MACF1	ENSG00000127603	9	0.12	0.61	Wnt signaling pathway
	ENSG00000102921	9	0.12	0.61	negative regulation of proteasomal ubiquitin-dependent protein catabolic process
ANTXR1	ENSG00000169604	9	0.12	0.61	actin cytoskeleton reorganization
CRTC3	ENSG00000140577	9	0.12	0.61	Transcription regulation. Coactivator of HTLV-1 Tax activation
ATG2B	ENSG00000066739	9	0.12	0.61	
RAB11FIP1	ENSG00000156675	8	0.12	0.64	Protein transport
SYT2	ENSG00000143858	8	0.12	0.64	neurotransmitter secretion
C17orf56	ENSG00000167302	8	0.12	0.64	Protein transport
ST8SIA3	ENSG00000177511	8	0.12	0.64	ganglioside biosynthetic process, N-glycan processing
MLL	ENSG00000118058	8	0.12	0.64	Apoptosis, transcription regulation
CYP2E1	ENSG00000130649	8	0.12	0.64	Metabolic process
BCAT1	ENSG00000060982	8	0.12	0.64	amino acid biosynthesis
ADCY9	ENSG00000162104	8	0.12	0.64	cAMP biosynthesis
THSD4	ENSG00000187720	8	0.12	0.64	Attenuates TGF- β signaling
FRMD4A	ENSG00000151474	8	0.12	0.64	
NSL1	ENSG00000117697	8	0.12	0.64	cell cycle, cell division
ARHGEF7	ENSG00000102606	8	0.12	0.64	neurogenesis
C2orf21	ENSG00000151989	8	0.12	0.64	
NCSTN	ENSG00000162736	8	0.12	0.64	Notch signaling pathway

	ENSG00000169116	8	0.12	0.64	positive regulation of telomerase activity
TSPAN9	ENSG00000011105	8	0.12	0.64	
MXD1	ENSG00000059728	8	0.12	0.64	transcription regulation
ZNF629	ENSG00000102870	8	0.12	0.64	transcription regulation
KIAA1715	ENSG00000144320	8	0.12	0.64	
	ENSG00000196758	8	0.12	0.64	
C13orf1	ENSG00000123178	8	0.12	0.64	
FMNL3	ENSG00000161791	8	0.12	0.64	actin cytoskeleton organization
BIRC4	ENSG00000101966	7.76	0.12	0.66	apoptosis, wnt signaling pathway
BACH2	ENSG00000112182	7.76	0.12	0.66	transcription regulation

^agenes in bold were found in both 5P and 3P targets

Table 5.2. BK polyomavirus 3P miRNA [bkv-miR-B1-3p] predicted targets.

Gene name ^a	Ensembl Gene ID	miTG score	Precision	SNR	Biological Function
DST	ENSG00000151914	18.68	0.85	1.06	Cell adhesion
ONECUT2	ENSG00000119547	12.68	0.07	1.06	Transcription regulation
ZNF468	ENSG00000204604	11.17	0.07	1.06	Transcription regulation
MACF1	ENSG00000127603	10.82	0.07	1.06	Wnt signaling pathway
ZC3H6	ENSG00000188177	10	0.07	1.06	
GJA7	ENSG00000182963	9.66	0.07	1.06	Cell communication
SYNGR1	ENSG00000100321	9.46	0.07	1.06	regulation of synaptic plasticity
MLL	ENSG00000118058	8.24	0.07	1.06	apoptosis, transcription regulation
TTN	ENSG00000155657	8.19	0.07	1.06	mitotic chromosome condensation, regulation of protein kinase activity
SNPH, RAD21L1	ENSG00000101298	8.15	0.07	0.53	synaptic vesicle docking involved in exocytosis
SCRN1	ENSG00000136193	8.09	0.07	0.56	exocytosis
KLF12	ENSG00000118922	8.05	0.07	0.6	Transcription regulation
KIAA1244	ENSG00000112379	8	0.07	0.79	negative regulation of phosphatase activity
PLEKHH2	ENSG00000152527	8	0.07	0.79	
ZNF490	ENSG00000188033	8	0.07	0.79	Transcription regulation
BMP8A	ENSG00000183682	8	0.07	0.79	cell differentiation
LRRIQ1	ENSG00000133640	8	0.07	0.79	
VPS13B	ENSG00000132549	8	0.07	0.79	protein transport
	ENSG00000154035	8	0.07	0.79	
LONRF2	ENSG00000170500	7.95	0.07	0.79	proteolysis
GIN1	ENSG00000145723	7.75	0.07	0.75	DNA integration
TSN	ENSG00000211460	7.66	0.07	0.73	DNA recombination
ITCH	ENSG00000078747	7.51	0.07	0.7	Antiviral defense, Apoptosis, Host-virus interaction, Innate Immunity
ZNF761	ENSG00000160336	7.51	0.07	0.7	
TMEM16C	ENSG00000134343	7.51	0.07	0.7	Ion transport
	ENSG00000196417	7.51	0.07	0.7	Transcription regulation
ACY1L2	ENSG00000146281	7.51	0.07	0.7	hydrolase activity
ALPK3	ENSG00000136383	7.51	0.07	0.7	heart development
FMNL3	ENSG00000161791	7.51	0.07	0.7	regulation of cell shape
ZNF283	ENSG00000176232	7.32	0.07	0.69	Transcription regulation

^agenes in bold were found in both 5P and 3P targets

early promoter in a strain-dependent manner (Abend et al., 2008); it is therefore conceivable that BKPyV may target TGF- β to regulate viral gene expression. Expression of another predicted gene target of the 5P miRNA, PXMP4, correlates with BKPyV viral titers (Schowalter et al., 2012). PXMP4 is peroxisome membrane protein 4, whose function is currently unknown. Indeed, there are many candidate targets whose functions are unknown which may have novel and undefined roles in viral infection.

As mentioned above, the 3P arm mature miRNAs are identical between BKPyV and JCPyV and therefore may share common cellular targets. However, miRNAs may also have unique targets that depend on cell specific expression. For example, JCPyV reactivates in the brain of progressive multifocal leukoencephalopathy patients, where it may have glial cell-specific targets. Another example of a cell-specific miRNA and virus interaction is that the host miRNA miR-122 is expressed specifically in liver, where it is required for maximal hepatitis C virus (HCV) RNA replication (Jopling et al., 2005). It is likely that host and viral miRNAs may be expressed in a cell-specific manner that can affect viral outcomes. Additionally, the host cell miRNA targets of BKPyV may be expressed in a cell-type specific manner. For instance, compared to RPTE cells, there may be unique cellular targets present in urothelial cells, which as discussed in the appendix, may be a site of viral persistence. It is also possible that host cell miRNAs may positively or negatively affect BKPyV infection, as is the case of miR-122 and HCV discussed earlier. Additionally, miRNA expression may be regulated differently, depending on cellular transcription factors present in a specific cell type, such that establishing a state of viral persistence in urothelial cells drives high miRNA expression that in turn downregulates viral replication and cellular factors contributing to viral replication. In Chapter

IV, we provide evidence that the miRNA may be controlled by unique regulatory elements within the NCCR compared to late transcripts.

In fact, it is likely that there are host cell factors, influenced by the BKPyV miRNA, able to regulate viral replication. In Chapter IV Figure 4.5D, we show that mutating the miRNA in the archetype virus increases replication but that the same mutation in the rearranged variant decreases replication. When no mature miRNA is produced in the miRNA mutant virus, levels of one or more unidentified cellular factors would increase and viral replication would decrease. Therefore, when not inhibited by the viral miRNA, such cellular factors may normally inhibit viral replication. Consequently, the miRNA may be directly or indirectly targeting a cellular factor that inhibits viral replication in both rearranged variant and archetype virus. However, this miRNA regulation of a potential cellular factor, potentially resulting in increased replication capacity, would not necessarily be appreciable in archetype virus, based on the dramatic role of the viral miRNA in limiting replication. Alternatively, archetype virus may have higher replication potential in the absence of both viral miRNA production and the potential regulatory cellular factor. It is also possible that targeting of this unidentified cellular factor which may promote viral replication only takes place during viral lytic replication such as rearranged variant replication in RPTE cells. This may further support the role of the miRNA as a key factor controlling the switch from persistence to lytic reactivation.

Another result noted in Chapter IV, Figure 4.5C, was that in addition to an increase in TAg protein expression there was also a difference in the ratio of the major TAg band compared to TAg*, the upper band, in the miRNA mutant vs. wt viruses. We believe TAg* may be a phosphorylated form of TAg. Indeed, there is evidence for the phosphorylation and slower migration of SV40 TAg (Bockus et al., 1987). Phosphorylated forms of TAg may have specific

cellular functions involved in viral replication. For example, in JCPyV, phosphorylation of TAg at threonine 125 binds more strongly to Rb pathway proteins and therefore may have a greater affect in regulating the cell cycle (Tyagarajan et al., 2006). In order to change the activity state of TAg, the BKPyV miRNA could potentially target the kinase responsible for its phosphorylation. Multiple kinases were identified as possible miRNA targets including PDPK1, PIP4K2B, PFKFB2. Although none of these kinases is known to directly phosphorylate polyomavirus TAg, PDPK1 regulates protein phosphatase (PP2Ac), which dephosphorylates hyperphosphorylated SV40 TAg to its active state (Aranda-Orgilles et al., 2011; Virshup et al., 1992). Together, this analysis shows that the BKPyV miRNA may regulate viral replication through both viral and host cellular targets.

To assess if there is selective pressure to maintain the integrity of the miRNA region, we looked for naturally occurring mutations and their frequency in and directly surrounding this region in BKPyV sequences in the NCBI database. At the time this analysis was completed (February 12, 2013) there were 298 sequences in the NCBI database; it is possible that some of these were duplicates. A total region of 270 nucleotides (nt) was analyzed for the number of sites mutated compared to Dik archetype virus from nucleotides 2611-2880 according to NCBI. The numbers of sites mutated in each region were as follows: upstream-late side (34/102 nt), 5P miRNA (1/22 nt), loop (1/16 nt), 3P miRNA (0/22 nt), downstream-early side (6/108 nt) (Figure 5.1A). The upstream region was subject to the most mutated sites, followed by the downstream region (Figure 5.1B). The downstream-early side is in the TAg coding sequence and is therefore likely conserved for TAg function. There was only one mutated site in the 5P miRNA that was present in only one variant, RYU-2. This site is not in the seed sequence which is required for

A

upstream late side
GGGTGGTGTT TTAGGCCTTT TAA AACATTG AAAGCCTTTA CACAAATGCA
* * * ^ *

ACTCTTGACT ATGGGGGTCT AACCTTTGGG AATCTTCAGC AGGGGCTGAA
* ** * * **** ^**** *** ***** * *

5P arm **loop** **3P arm**
GTATCTGAGA CTTGGGAAGA GCATTGTGAT TGGGATTCAG TGCTTGATCC
* * *

downstream early side
ATGTCCAGAG TCTTCAGTTT CTGAATCTTC TTCTCTTGTG ATATCAAGAA
^ * ^ **

TACATTTCCC CATGCATATA TTATATTTCA TCCTTGAAAA AGTATACATA
*

CTTATCTCAG AATCCAGCCT

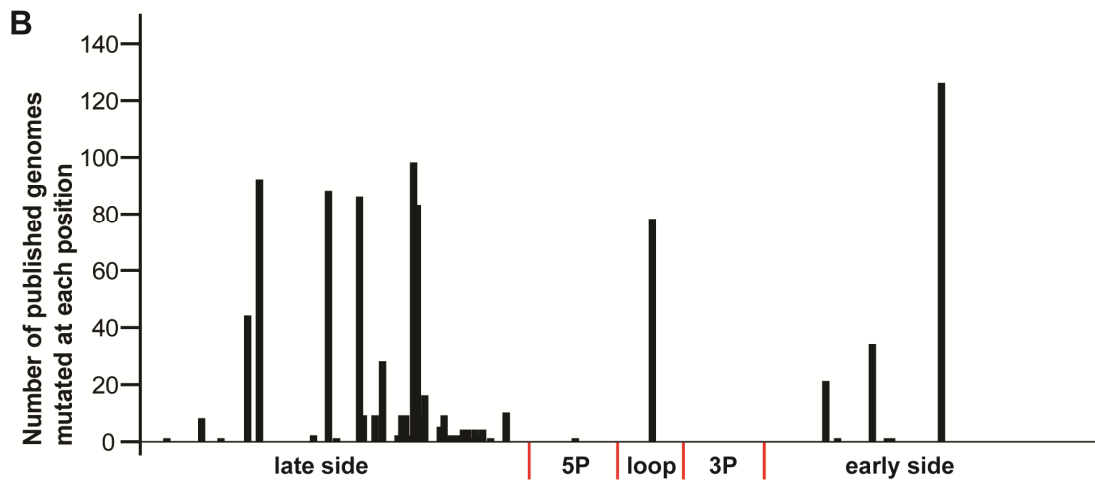


Figure 5.1. Naturally occurring mutations in the BKPyV miRNA region. (A) Sequence of the 270 nt region surrounding the BKPyV miRNA using Dik archetype virus as a reference. The 5P and 3P arm mature miRNAs are underlined. *, position of a naturally occurring mutation; ^, position mutated in the rearranged variant Dunlop. (B) Graph of the number of published genomes mutated at each position. A total of 298 published genomes were analyzed. The y axis is nt 1-270 and divided by region with the solid red lines below the x axis. late side, sequence upstream of the miRNA near the late transcript; 5P, 5P miRNA; loop, loop between mature miRNAs; 3P, 3P miRNA; early side, sequence downstream of the miRNA in the early transcript coding region.

miRNA function, so the 5P miRNA is likely still able to functionally target early mRNA and any potential cellular targets. These results show that the miRNA region is highly conserved, compared to the surrounding region, which implies that it serves an important function during infection.

The differences between the archetype virus and rearranged variant particle

Archetype virus is the transmissible form of BKPyV that establishes a persistent infection in the host urinary tract. It is also the form most commonly isolated both from healthy people as a result of periodic reactivation and from immunosuppressed patients (Knowles, 2001). However, prior to the work performed in the dissertation, it was not possible to propagate archetype BKPyV in culture. As discussed in Chapter III, archetype virus propagation can be induced in culture by overexpressing TAg. We were able to grow and purify archetype virus from 293TT cell, which highly express SV40 TAg (Broekema et al., 2012). We observed that purified archetype virus produced a statistically significantly smaller particle measuring 44.9 ± 2.0 nm ($n = 85$), compared to the rearranged variant particle measuring 46.6 ± 2.5 nm ($n = 34$) ($p < 0.001$). Additionally, we noted that archetype virus was more infectious in 293TT cells than a rearranged variant by measuring the genomes to infectious unit (IU) ratio. The genome number was measured by quantitative PCR of encapsidated viral genome while the viral titer or IU was measured by fluorescence focus assay for VP1 protein expression in 293TT cells. Archetype virus had an average genome/IU ratio of 1352 while the rearranged variant has 6875 genomes/IU. Therefore archetype virus is approximately five times more infectious compared to rearranged variant; i.e., it would take five times fewer genomes to establish an infection for archetype virus. We also noted that archetype virus and not rearranged variant had an increased

rate of loss of infectivity when stored at -80 °C. The titer of purified archetype virus dropped by almost 1 log unit after 16 months, compared to 0.3 log unit for rearranged variant after 22 months. This suggests that there are inherent differences in the archetype virus particle compared to the rearranged variant that may contribute toward the differences in culture replication phenotype.

One possible explanation for the difference between archetype virus and rearranged variant particles is a difference in chromatin packaging. The BKPyV genome is packaged as a minichromosome, a cellular chromatin-like structure, within the capsid and functions as the template for transcription (Meneguzzi et al., 1978). The archetype viral genome may be in a more heterochromatin or transcriptionally-inactive state as a result of differential methylation or acetylation within the NCCR [reviewed in (Bhaumik et al., 2007)]. For example, genes that have been silenced are typically associated with hypoacetylated histones. Possible histone modifications include acetylation, methylation, ubiquitination, and phosphorylation. Each of these modifications has the potential to alter the interaction between the DNA and histone, which can alter the structure of the minichromosome. The archetype virus chromatin may be differentially modified, resulting in an increased heterochromatin structure, which is associated with higher chromatin compaction (Dillon, 2004), possibly leading to a smaller particle size. As discussed in Chapter IV, archetype virus is unable to replicate upon infection of RPTE cells as a result of a balance of regulatory elements within the NCCR, resulting in high miRNA expression relative to its target early mRNA, which is required for viral DNA replication. We also presented evidence that the miRNA may be regulated by unique elements on the late strand in the NCCR. It is possible that the unidentified miRNA promoter may not be subject to heterochromatin formation and may thereby continue to be expressed at a relatively high level in the archetype

virus. However, gene silencing as a result of heterochromatin condensation is another factor that could contribute to the inability of archetype virus to replicate in many different cell types. A transcriptionally-inactive state may also promote persistence of archetype virus by limiting virus progeny production. Therefore, it will be important to investigate differences in epigenetic marks between archetype virus and rearranged variants, specifically within the NCCR, the major region controlling replication. If differential heterochromatin induction is involved in the mechanism of establishing or maintaining viral persistence, it may give us clues for drugs that could promote lytic reactivation in a cell culture model of latency. For example, histone deacetylases (HDACs) are enzymes that promote chromatin condensation by removing acetyl groups from lysine residues. Therefore, it would be interesting to test if an HDAC inhibitor, such as sodium butyrate, could promote lytic reactivation in a model of archetype virus persistence.

Conclusions

Based on the work described in this thesis and the discussion in this chapter, we have proposed a model of archetype virus persistence and reactivation detailed in Figure 5.2. Archetype virus likely establishes persistence as an episome in the human urinary tract, possibly in urothelial cells. Viral and host immune factors contribute to (a) controlling viral replication, (b) limiting viral spread, or (c) promoting cell survival. Any or all of these mechanisms are potentially involved in establishing and/or maintaining viral persistence. Recently, our laboratory and others have described the host immune contribution toward regulating viral replication that likely plays a role in maintaining persistence. The DDR pathway, an intrinsic signaling pathway, is important in promoting survival of BKPyV infected cells (Jiang et al., 2012). Innate immune factors are critical in controlling replication and viral spread. The cytokine interferon- γ inhibits

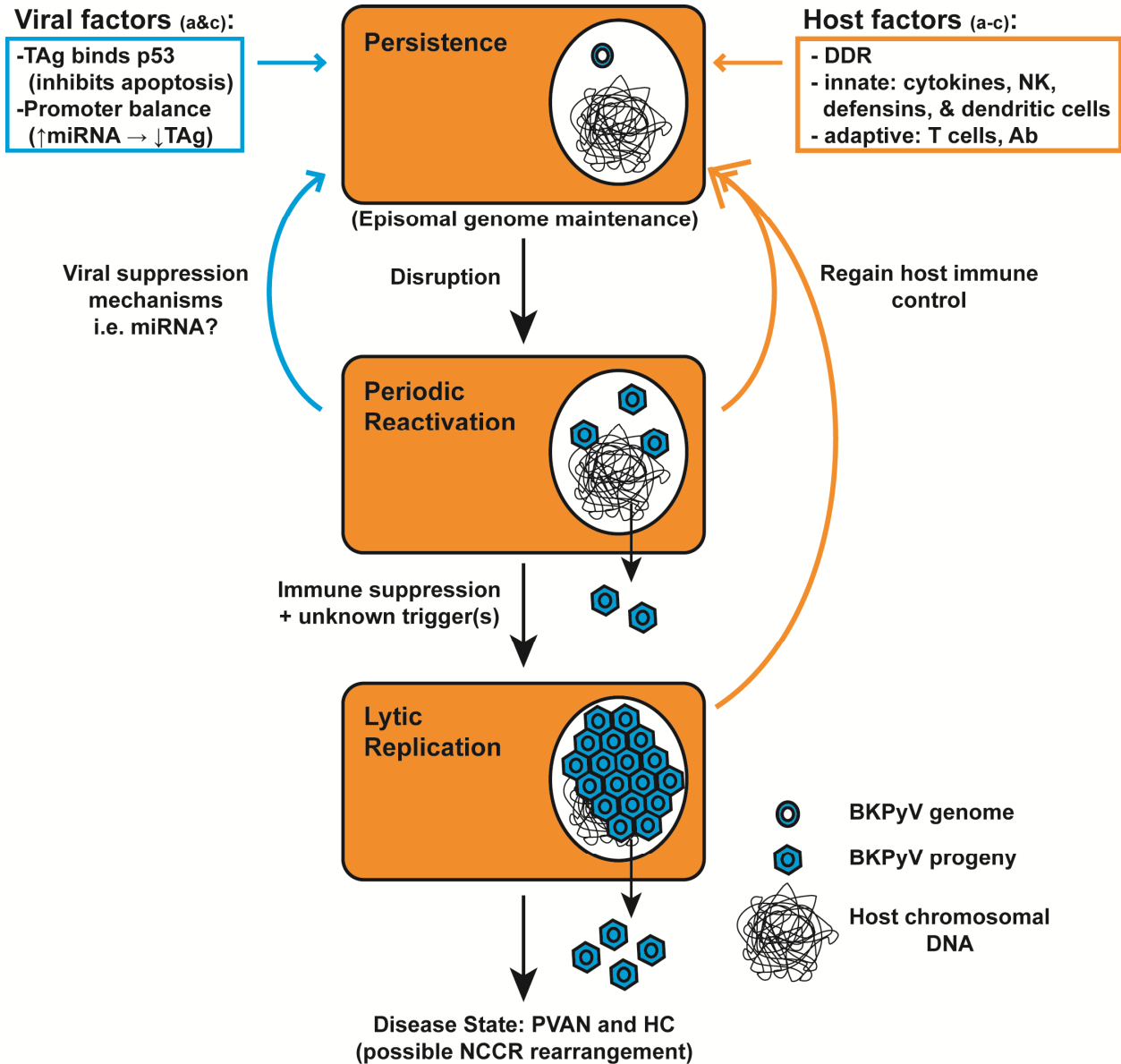


Figure 5.2. Overview model of potential factors contributing to BKPyV persistence. Both viral and cellular factors contribute to (a) limiting viral replication, (b) limiting virus spread, and/or (c) promoting cell survival; each of which could potentially contribute to viral persistence within the host. The virus likely persists in the cell as an episome at an unknown copy number and can periodically reactivate and produce progeny through an undefined mechanism. At this point both viral and host mechanisms may contribute to re-establishment of persistence. In the case of transplant patients, immune suppression in combination with unknown triggers promotes viral lytic replication which can resolve to a state of overall persistence, likely a result of host immune factors, or progress to a disease state and possible NCCR rearrangement.

viral transcription and replication (Abend et al., 2007), which could promote long-term persistent infection of the cell and host. There is suggestive evidence for the role of NK cells in limiting BKPyV, in that the 3P miRNA targets ULBP3, which is recognized by NKG2D, a receptor for NK cells (Bauman et al., 2011). Additionally, NK cells are producers of interferon- γ ; therefore they could play a role in controlling viral replication and limiting viral spread through immune recognition. Defensins reduce cellular attachment of the virus by causing aggregation, thereby limiting viral spread (Dugan et al., 2008). Patients with reduced number of dendritic cells, which are important for antigen presentation, are at increased risk of developing BKPyV viremia (Babel et al., 2011). Lastly, adaptive immune factors such as T cells and neutralizing antibodies may limit viral spread to promote persistence (Chen et al., 2006; Pastrana et al., 2012).

Importantly, the results described in this dissertation add a role for viral factors that may contribute toward establishment or maintenance of persistence. The archetype virus balance of NCCR regulatory elements leads to high levels of miRNA expression relative to low levels of its target early mRNA, which inhibits viral replication. Another previously described interaction between viral-host cell factors that may contribute to viral persistence is TAg binding p53 to inhibit apoptosis, thereby promoting cell survival (Bocchetta et al., 2000; Harris et al., 1996). Through an unknown mechanism, archetype virus can overcome these mechanisms of regulation and produce progeny during periodic reactivation in healthy people. Following immune suppression, such as in transplant patients, and in combination with unknown triggers, the virus proceeds to lytic replication that can progress to a diseased state such as PVAN or HC, which may be accompanied by possible NCCR rearrangement. The mechanism of NCCR rearrangement is currently unknown. One possibility is that once induced, archetype viral DNA replication leads to an accumulation of genomes within the nucleus of an infected cell which

then serve as a template for homologous recombination. Homologous recombination between genomes could then result in duplications and deletions of sequence within the NCCR.

Rearrangements that enhance replication ability could then be selected for in a patient where viral DNA replication remains unchecked. Since archetype virus is thought to persist and replicate mainly in the urothelium, rearrangements may be selected for that enhance the ability to replicate at other locations, such the proximal tubule of the kidney. This would then allow rearranged variants direct access to the bloodstream, possibly explaining why these variants are more frequently isolated from serum of patients with BKPyV-associated disease, whereas archetype virus is commonly isolated from the urine.

In conclusion, the work described in this dissertation has advanced our understanding of archetype BKPyV. We have shown that the persistent form, archetype virus, uniquely regulates its replication through differential expression of the viral miRNA and its target early mRNA as compared with rearranged variant. This expression is a result of a balance of regulatory activities within the NCCR and results in limiting viral replication in the archetype virus. We also provide preliminary data on urothelial cells as a model of archetype virus persistence. Finally, we discuss future areas of study for archetype BKPyV. There is no specific antiviral treatment for BKPyV-associated disease, therefore it is important to better understand both the viral and host factors that contribute toward the mechanism of viral persistence, which may advance treatments to prevent reactivation or inhibit replication of reactivated virus in transplant patients.

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Appendix

Preliminary studies of archetype virus persistence in urothelial cells

Introduction

Polyomaviruses exist in two forms, designated archetype virus and rearranged variants, which are differentiated by the structure of their non-coding control region (NCCR) genomic DNA. The archetype virus NCCR DNA sequence is divided into five blocks designated O, P, Q, R, and S. Rearranged polyomavirus variants have deletions and/or duplications in this block structure and readily replicate in many cell types, including primary renal proximal tubule (RPTE) cells, a natural cell culture model of lytic replication (Low et al., 2004). Rearranged variants are found almost exclusively in BKPyV-associated disease such as polyomavirus-associated nephropathy (PVAN) or hemorrhagic cystitis, in renal and bone marrow transplant (BMT) recipients, respectively. However, no natural cell culture model of archetype virus persistence or replication has yet been developed for any human polyomavirus. Despite this, archetype virus is the most commonly isolated form of BKPyV and can be detected at high titer in the urine of diseased patients and healthy people as a result of transient reactivation events (Knowles, 2001). Therefore, there must be cell(s) that are able to support persistence and/or propagation of archetype virus within the host. We set out to identify a natural host cell culture model for archetype virus persistence and reactivation.

Archetype virus is thought to be the transmissible form of the virus since it can be isolated from both healthy individuals and diseased patients. Additionally, its NCCR structure

contains all the genetic information necessary to give rise to rearranged variants. Most of the previous attempts to propagate archetype polyomaviruses over long term passage in cell culture have resulted in selection for rearrangements in NCCR structure (O'Neill et al., 2003; Rubinstein et al., 1991; Sundsfjord et al., 1990). However, there is one report of archetype BKPyV propagation in cell culture. Archetype virus had extremely limited replication capacity when transfected into a human endothelial cell line (Hanssen Rinaldo et al., 2005). Infectious progeny were not detected until 35 days post transfection (dpt), and only 4% of cells were infected upon secondary infection of human endothelial cells with transfection lysates. While some of the virus produced maintained the archetype virus NCCR configuration, there were also a significant number of rearranged variants detected. It is unclear whether rearrangement of the NCCR was selected for upon reinfection of endothelial cells. Therefore, this system is very inefficient for archetype BKPyV propagation. In addition, since endothelial cells are not a natural host cell for BKPyV, this is not an ideal cell culture system to study the biology of the virus. Consequently, a natural host cell model of archetype virus persistence and reactivation is needed to determine the mechanisms underlying these stages of the viral lifecycle.

Viral persistence is a long-term infection that is not cleared by the host. Viral latency, a form of persistence, is defined by three characteristics: (1) maintenance of the viral genome, (2) the absence of progeny production, and (3) a stimulus of viral reactivation is required to confirm that the DNA persisting in the cell maintains its infectivity (Boldogh et al., 1996; Speck et al., 2010). The best studied viruses that undergo true latency are the herpesviruses. Gene transcription is limited during herpesvirus latency to genes and miRNAs that suppress the viral regulator of lytic reactivation, direct episomal replication and segregation, and modulate host signaling (Grey et al., 2007; Murphy et al., 2008). A different type of viral persistence is seen for

human papillomavirus (HPV), small DNA viruses related to polyomaviruses. A model of HPV persistence has been developed using human foreskin keratinocyte (HFK) cells (Frattoni et al., 1997). In this model, recircularized viral DNA is transfected into HFK cells with a plasmid expressing a selectable marker. HPV DNA is maintained episomally in these cells and replication is induced upon cellular differentiation in raft cultures. The raft culture is designed to promote growth of cells that mimic their function and morphology *in vivo*. These cells are grown on collagen plugs and are maintained at a liquid-air interface (Frattoni et al., 1996). Viral progeny are only assembled in the upper granular layer and released from the cornified layer of the stratified cell culture model (Moody et al., 2010). Unlike HPV, both herpesviruses and polyomaviruses are known to produce autoregulatory miRNA(s), which likely play a role in viral persistence (Murphy et al., 2008). At this point, it is not clear whether BKPyV is able to establish a true latent infection, a more general persistent infection akin to HPV, or a form of persistence combining factors from both kinds of viruses.

In these preliminary studies we have investigated urothelial cells as a potential model of archetype BKPyV persistence. The urothelium, like keratinocytes, form a true stratified epithelium that contains basal, intermediate, and superficial cells (Jost et al., 1989). These cells are polarized and display increasing differentiation complexity from the basal layer to the surface. In this appendix we show that the virus is specifically maintained over extended passaging of urothelial cells in monolayer culture and that no viral progeny are produced from these cells.

Urothelial cells maintained in defined low serum and calcium media maintain a proliferative undifferentiated phenotype (Southgate et al., 1994). In the future, we plan to establish a transwell model of stratified urothelial cells in the presence of high serum and

calcium to examine the potential role of differentiation in archetype virus reactivation, similar to what is seen for HPV in keratinocytes.

Materials and methods

Cell culture. Urothelial cells immortalized with HPV E6 and E7 (Carmean et al., 2007) were obtained from Monica Liebert at the University of Michigan and were maintained in EpiLife media (Gibco) with 1% human keratinocyte growth supplement, 100 U/ml penicillin, and 100 µg/ml streptomycin. 293TT cells were obtained from Chris Buck at the National Cancer Institute and were maintained under 400 µg/ml hygromycin selection (Buck et al., 2004) in Dulbecco's modified Eagle medium with 10% fetal bovine serum and 100 U/ml penicillin, 100 µg/ml streptomycin. All cells were grown in a 5% CO₂ environment in a humidified 37°C incubator.

Constructs. The pBR322-Dunlop plasmid (ATCC #45025, pBKV) contains a full length Dunlop (rearranged NCCR) genome (Seif et al., 1979) and the pBR322-Dik plasmid contains a full length Dik (archetype NCCR) genome (Nishimoto et al., 2006). The Dik and Dunlop genomes each were subcloned from these plasmids into pGEM7zf(+) at the *Bam*HI site to facilitate plasmid growth as a high copy vector. The empty vector genome was pGEM7zf(+).

pcDNA3.1+hygro was co-transfected with the recircularized viral genome or empty vector.

Transfection, Infection and Western blotting. Recircularized archetype virus (Dik), rearranged variant (Dunlop) genome, or a linearized empty vector were co-transfected with pcDNA3.1+hygro into urothelial cells at 100% confluency using TransIT LT-1 transfection reagent (Mirus Bio, Madison, WI) according to the manufacturer's instructions as described previously (Broekema et al., 2010). Transfection complexes were removed 18 h post-transfection by washing once with PBS and adding fresh media. E1A lysis buffer (Harlow et al., 1986) was

used to harvest total cell proteins, which were analyzed as previously described (Jiang et al., 2009).

PCR and quantitative PCR. To detect episomally maintained viral DNA in urothelial cells, low molecular weight DNA was isolated using a Hirt isolation protocol (Hirt, 1967). PCR was performed using Invitrogen Taq DNA polymerase according to the manufacturer's instructions. A region of viral DNA surrounding the NCCR was amplified using primers 5'-TTTGTCAGGGTGAAATTCCTT-3' and 5'-TCACCTCTACAAAATTCCAGCA-3'. The ampicillin resistance gene in the empty vector was amplified using primers 5'-GAGTATTCAACATTTCCGTGTCGCC-3' and 5'-CCAATGCTTAATCAGTGAGGCACC-3'. Quantitative PCR (qPCR) was performed to quantify episomally maintained viral DNA, and the data were analyzed as previously described (Jiang et al., 2011). Viral DNA was normalized to "input DNA" harvested at passage 1 (2 dpi) using a Δ Ct method.

Results and Discussion

BKPyV TAg has been detected in urothelial cells in patient samples by immunohistochemistry (Roberts et al., 2008). Additionally, morphologically distinct infected urothelial cells that are shed in the urine, called decoy cells, have long been used as a diagnostic test for BKPyV infection (Ahsan et al., 2006; Kahan et al., 1980). Therefore, we hypothesize that urinary tract cell types such as urothelial cells may support archetype BKPyV persistence and replication under proper stimulatory conditions. To examine the episomal maintenance of viral DNA, urothelial cells were transfected with recircularized archetype virus DNA, and low molecular weight DNA was harvested at each cell passage (Hirt, 1967). Only episomally-maintained DNA is obtained via low molecular weight DNA isolation since cellular DNA is

discarded during this procedure. The presence of viral DNA was assayed at each cell passage by quantitative PCR (qPCR) (Figure A.1). Untransfected urothelial cells had no detectable viral DNA. Archetype viral DNA was detected out to 45 dpt but was maintained at a low level. Urothelial cells were found to have low transfection efficiency (less than 10%), so it is possible that cells transfected with the viral genome were diluted by untransfected cells that continued to replicate. In a parallel experiment, urothelial cells were transfected with rearranged variant genome and were found to have delayed kinetics of replication compared to transfection of RPTE cells (see Chapter II). Viral DNA declined and then increased, and after cell passage 6 (27 dpt), all the cells died as a result of lytic infection. Viral progeny were detected by fluorescence focus assay in whole cell lysates harvested after passage 6 from cells transfected with the rearranged variant but not the archetype virus.

In order to avoid the possible dilution of viral genomes in transfected cells, we next repeated this experiment in the presence of antibiotic selection. Urothelial cells were cotransfected with a plasmid encoding a hygromycin B resistance gene and either the recircularized archetype viral genome or empty vector. We then maintained cells under selection and, as before, harvested low molecular weight DNA during cell passaging. Viral DNA was detected by PCR out to 71 dpt; however, the signal declined at each passage tested, indicating that the urothelial cells were not maintaining the genome at a constant level (Figure A.2). As a control, urothelial cells were transfected with a linearized empty vector and a plasmid encoding hygromycin B resistance. This plasmid was not detected after 25 dpt, indicating that viral DNA is specifically maintained in urothelial cells. In the future, these experiments will be repeated with a selected clonal population of transfected urothelial cells to determine if starting viral genome copy number can be maintained in a clonal population of cells. To generate a clonal

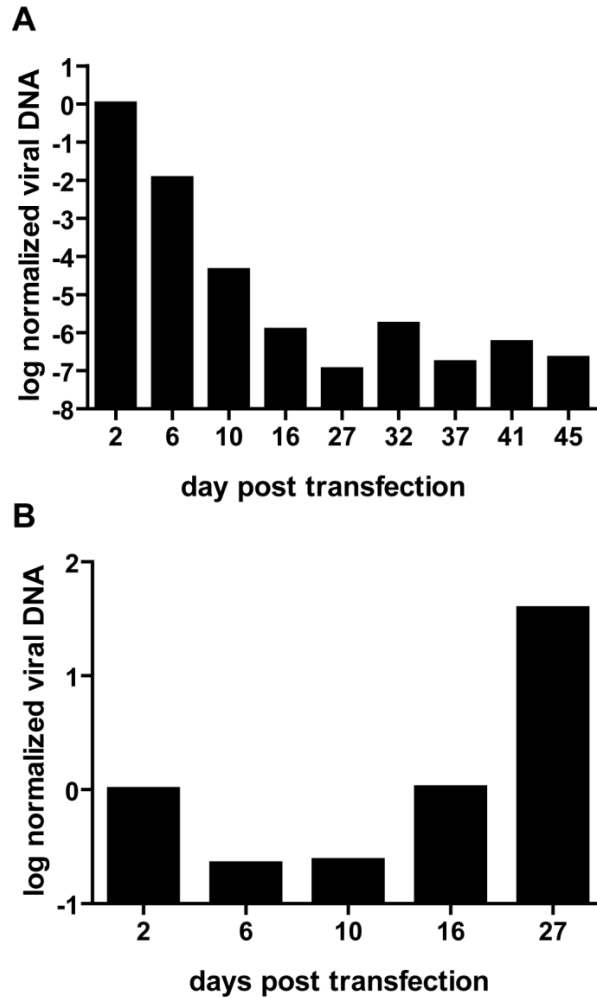


Figure A.1. Archetype virus DNA is maintained as an episome in urothelial cells. Urothelial cells were transfected with recircularized (A) archetype virus (Dik) or (B) rearranged variant (Dunlop). Low molecular weight was harvested at each cell passage and analyzed by qPCR for the presence of viral DNA. Results from one experiment are presented as log Δ Ct where 2 dpt (cell passage 1) is arbitrarily set to one.

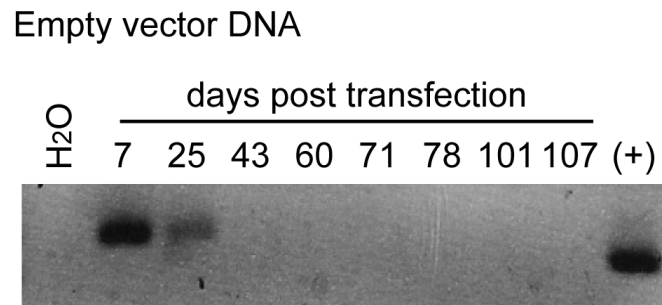
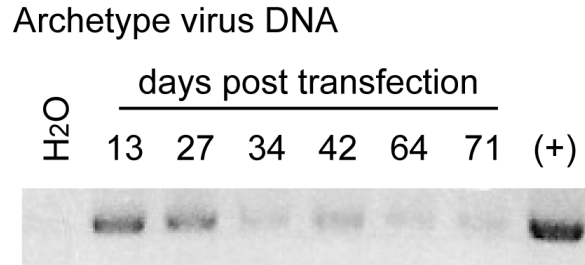


Figure A.2. Archetype virus DNA is specifically maintained as an episome in urothelial cells under selection. Urothelial cells were co-transfected with a plasmid encoding a hygromycin resistance gene and either recircularized archetype virus (Dik) or empty vector. Low molecular weight DNA was harvested at designated cell passages and analyzed by PCR for the presence of archetype virus DNA (upper) or empty vector DNA (lower).

population, cells will be transfected with recircularized viral genome and a plasmid encoding hygromycin B resistance. For example, single cell clones could be generated by limiting dilution into a 96 well plate and growth under hygromycin selection. Clones will then be screened for the presence of viral DNA by qPCR.

In addition to genome maintenance, lack of progeny production is another defining characteristic of viral persistence. VP1 capsid protein production is an indirect measure of lytic replication and progeny production. Total cellular protein was analyzed at each cell passage by Western blot analysis. No capsid protein production was detected in archetype virus transfected cells.

To directly monitor any potential progeny production, 293TT cells were used to amplify progeny produced from transfected urothelial cells. We previously showed that 293TT cells support the propagation of both archetype and rearranged variants (Broekema et al., 2012). Total cellular lysates were collected at each passage and used to infect 293TT cells, and total cellular proteins were then harvested 5 dpi. Western blotting was performed for the VP1 capsid protein. Urothelial cells transfected with the archetype virus genome did not produce detectable progeny, as measured by secondary infection of 293TT cells. However, rearranged variant transfected cells that were killed by passage 6 due to lytic replication did produce VP1 in 293TT cells, indicating the presence of infectious progeny.

Prior to our development of a system for the propagation of archetype virus in 293TT cells, all previous attempts to propagate archetype virus in culture have resulted in selection for NCCR rearrangements (Broekema et al., 2012; Hanssen Rinaldo et al., 2005; Rubinstein et al., 1991). We hypothesize that a natural cell culture model of archetype virus persistence will support maintenance of the archetype NCCR structure. To confirm that rearrangements are not

selected for in urothelial cells, the NCCR will be PCR amplified and cloned from the DNA harvested at each passage. At least 26 clones per passage will then be sequence. Using the Wilson's confidence interval for binomial proportion: if all 26 clones are archetype in sequence then the true proportion of rearranged variants is below 10%, with 95% confidence. I expect that archetype virus will be maintained as an episome in urothelial cells *in vitro* and will not produce viral progeny under normal cellular conditions.

In this appendix we present preliminary data that archetype BKPyV viral DNA is specifically maintained over extended passaging in urothelial cells in monolayer culture and that progeny are not produced from these cells. These results support the hypothesis that urothelial cells are a potential model of archetype viral persistence, although more work is needed to confirm that viral DNA can be maintained long term and that it remains infectious. Areas of future research and considerations for a cell culture model of viral persistence are discussed in Chapter V.

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