

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

Nicole M. Broekema^{a,e,1}, Johanna R. Abend^{a,1,2}, Shauna M. Bennett^{b,e}, Janet S. Butel^c, John A. Vanchiere^d, Michael J. Imperiale^{a,b,e,*}

^a Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, MI 48109-5620, USA

^b Program in Cellular and Molecular Biology, University of Michigan Medical School, Ann Arbor, MI 48109, USA

^c Department of Molecular Virology and Microbiology, Baylor College of Medicine, USA

^d Department of Pediatrics, Louisiana State University Health Sciences Center, Shreveport, USA

^e Comprehensive Cancer Center University of Michigan Medical School, Ann Arbor, MI 48109, USA

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ABSTRACT

The human polyomavirus BK virus (BKV) is an important opportunistic 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. BKV exists as archetype virus and rearranged variants, which are classified based on the DNA sequence of their non-coding control regions (NCCRs). The archetype BKV 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 BKV. 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 BKV replication ability *in vitro*.

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Introduction

BK virus (BKV) is a highly prevalent human polyomavirus originally isolated from the urine of a renal transplant patient with ureteric obstruction (Gardner et al., 1971). BKV is believed to be spread asymptotically by a respiratory route, based on evidence that seroconversion takes place in early childhood and that BKV 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., 2005a, 2009; Wong et al., 2009). The seroprevalence of BKV in the population is reached by the age of 10 and is estimated to be between 65% and 90% (Kean et al., 2009; Knowles, 2006). Following primary infection, BKV establishes a lifelong, persistent, subclinical infection in the urinary tract (Chesters et al., 1983; Heritage et al., 1981). Renal tubule epithelial cells are a major site in which BKV persists and can be reactivated (Knowles, 2001; Moens and Rekvig, 2001). BKV 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, BKV can reactivate and cause diseases such as hemorrhagic cystitis (HC) in bone marrow transplant patients and polyomavirus-associated nephropathy (PVAN) in kidney transplant patients. BKV 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, BKV infection in HIV/AIDS patients can result in death (Cubukcu-Dimopulo et al., 2000).

BKV 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). BKV 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 BKV is characterized by five blocks of DNA sequence within the NCCR: O, P, Q, R, and S (Fig. 1A). The O block contains the origin of replication and putative transcription factor binding sites. Rearranged variants of BKV 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

* Corresponding author. 1150 West Medical Center Drive, 5724 Medical Science II, Ann Arbor, MI 48109-5942, USA. Fax: +1 734 615 6560.

E-mail addresses: broekem@umich.edu (N.M. Broekema), johanna.abend@nih.gov (J.R. Abend), shaunamb@umich.edu (S.M. Bennett), jbutel@bcm.edu (J.S. Butel), jvanch@lsuhsc.edu (J.A. Vanchiere), imperial@umich.edu (M.J. Imperiale).

¹ These authors contributed equally to this work.

² Present address: HIV & AIDS Malignancy Branch, NCI, NIH, Bethesda, MD 20892 USA.

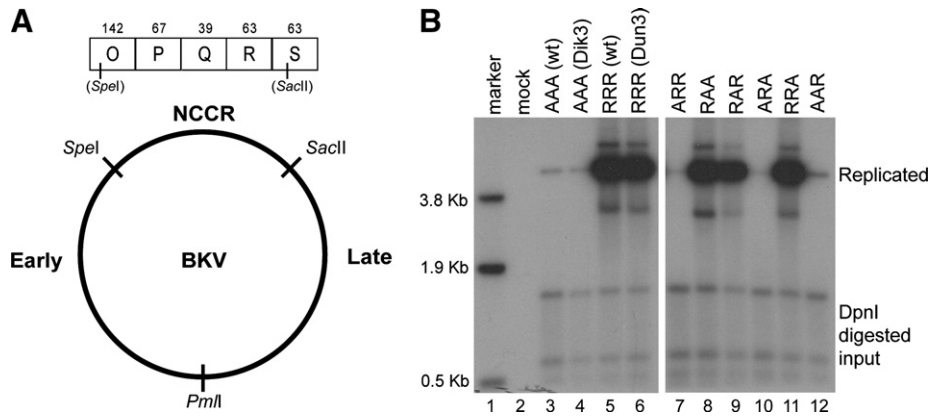


Fig. 1. The NCCR determines replication efficiency of BKV. (A) Schematic of the swap genome with the archetype virus (Dik) NCCR. *SpeI* and *SacII* sites were inserted into the pBR322-Dunlop or -Dik vectors flanking the majority of the NCCR and a *PmlI* site was inserted between the early and late regions. (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.

transcriptional enhancers, as defined in rearranged variants, are located in the P, Q, R, and S blocks (Markowitz and Dynan, 1988; Moens et al., 1995; Moens and Van Ghelue, 2005). BKV archetype virus can be isolated from both healthy individuals and diseased patients, and it currently cannot be grown in cell culture. Rearranged variants are most commonly isolated from patients with BKV 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 BKV, when high titers of virus are present in the serum of a patient with BKV disease, the appearance of rearranged variants increases and these eventually replace archetype as the predominant form of the virus (Gosert et al., 2008). 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., 2008). A recent study showed that multiple rearranged quasispecies can be found in the urine or allograft biopsies of kidney transplant recipients, even 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 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 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 BKV 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 BKV 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 BKV viruria. 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 BKV replication.

Results

The NCCR is sufficient to determine replication ability in cell culture

BKV 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 BKV 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 (Fig. 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, recircularized, and transfected into RPTE cells. At 5 days post-transfection, low molecular weight DNA was isolated (Hirt, 1967), linearized, digested with *DpnI*, and assayed by Southern blotting (Fig. 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 (Fig. 3C and data not shown). 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 BKV NCCR variants isolated from an immunocompromised patient

To test the utility of this system for analyzing patient isolates, we cloned the BKV NCCR from a urine sample of an AIDS patient with BKV viraemia. The serum and the urine of this patient were tested for the presence of BKV and JCV by quantitative PCR as previously described (McNees et al., 2005). JCV was not detected in either fluid and BKV was only detected in the urine, at 150 genomes/ml. The BKV amplicons from the patient were sequenced and classified as genotype Ia based on VP1 polymorphisms (Jin et al., 1993; Zheng et al., 2007). We were able to isolate five distinct NCCRs from this single urine specimen displaying differing degrees of rearrangement (Fig. 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 (Fig. 3A). Day 4 was chosen because parental rearranged variant viral replication can be easily detected at this time point (data not shown). To quantify replication ability, the replicated genome band was normalized to a *DpnI*-digested input band (Fig. 3A,

arrow) to control for transfection efficiency and the archetype virus (Dik3) was set to one (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 4).

Discussion

The determinants that regulate the ability of archetype virus and rearranged variants of BKV to replicate in cell culture have not yet been systematically mapped. In human embryonic kidney and adult human skin fibroblast cells, 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 BKV 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 BKV 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 BKV 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 and Fanning, 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

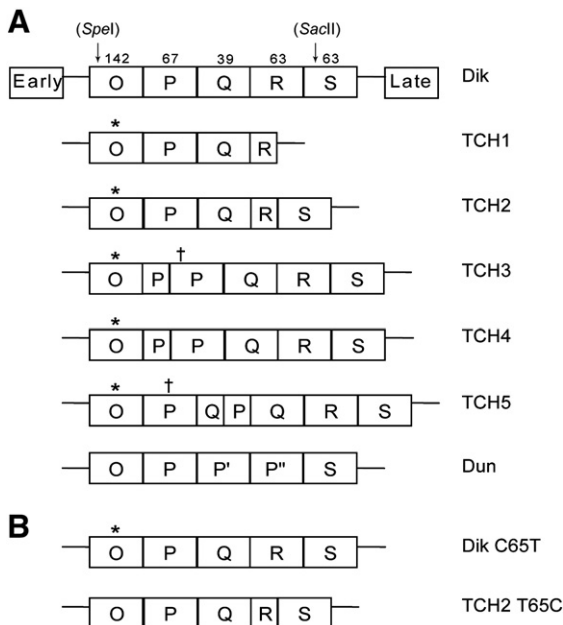


Fig. 2. (A) Schematic of the NCCRs of viruses isolated from a single clinical specimen. The NCCR is divided into blocks of sequence O, P, Q, R, and S. The block structures of the archetype virus (Dik), the rearranged variant (Dun), and the variants isolated from the patient are shown. The * represents 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.

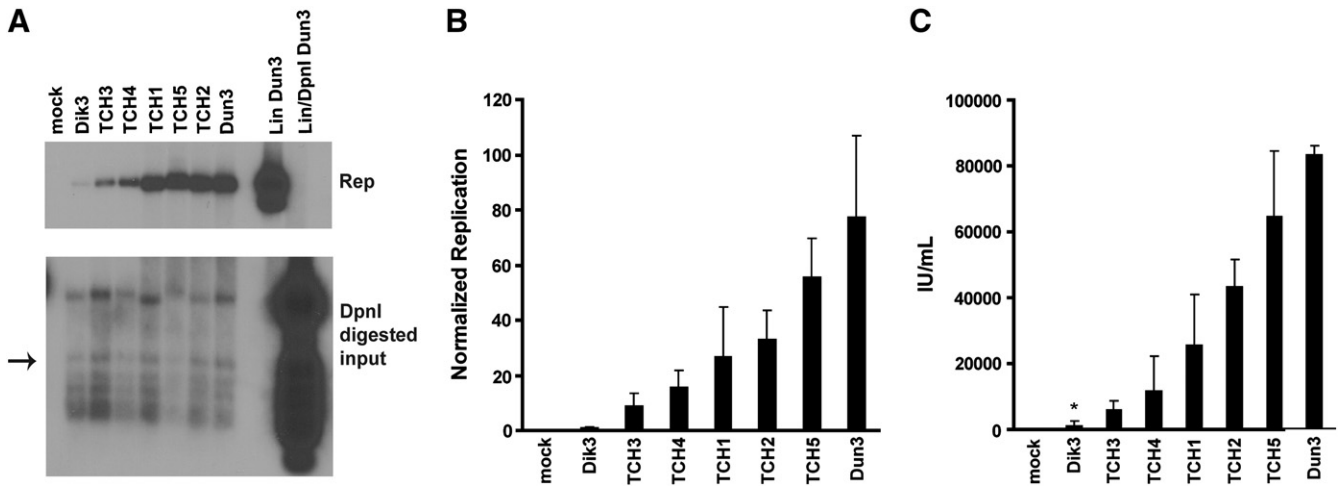


Fig. 3. Clones isolated from a clinical specimen display variable replication abilities. RPTE cells were transfected with recircularized viral genomes. (A, B) Low molecular weight DNA was harvested 4 dpt and analyzed as in Fig. 1. (A). Representative Southern blot. The two panels are different exposures 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 Materials and methods. Results are from three independent experiments, error bars represent standard error of the mean. The * represents a measurement below the limit of detection.

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 BKV NCCRs cloned directly from a single urine specimen from an AIDS patient with BKV 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 BKV variants in serum is associated with increased replication and inflammation (Gosert et al., 2008). While virus excreted in the urine may be produced at a different site of replication, 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 BKV 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 BKV 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 BKV 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 BKV NCCR as well (Del Vecchio et al., 1989; Yang and Wu, 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 and Subramani, 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 BKV variants in

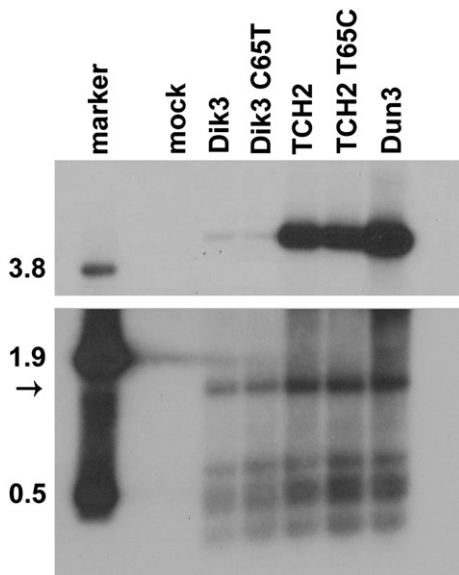


Fig. 4. The O block polymorphism does not affect replication ability. The C65T mutation was created and reversed in the O blocks of Dik3 and TCH2, respectively, and the genomes were assayed as in Fig. 3A. The blot is representative of two independent repeats, and the two panels are different exposures of the same blot. Marker: *HindIII* digest of pGEM7-TU (size of bands in kb); Mock, mock transfection.

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 Lednický. Both of these genomes are inserted into the pBR322 backbone at the *Bam*HI site. Site-directed mutagenesis was performed using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene) as described previously (Abend and Imperiale, 2008) on both pBR322-Dik and Dunlop to create Dik-3-site (Dik3) and Dunlop-3-site (Dun3) swap vectors. The *Spe*I site was inserted into the O block using primer A and its reverse complement and the *Sac*II site was inserted using primer B and its reverse complement (Table 1). The *Pml*I site was inserted between the early and late regions 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 1). Mutations were confirmed by DNA sequencing. The integrity of the early coding region was also confirmed by DNA sequencing.

Table 1
List of primers used for site-directed mutagenesis and SOEing PCR^a.

Primer	Sequence (5'–3')
A NCCR early <i>Spe</i> I	GGGGAATCACTAGTCTTTTGCAATTTTGCAAAAATGG
B NCCR late <i>Sac</i> II	GACAAGGCCAAGATTCCGGCGCTCGAAAACATGTC
C Tag <i>Pml</i> I	ACACCACCCCAAAATAACACGCTGCTTAAAGTGGCTTATAC
D NCCR bridge For	TAGTAAGGGTGTGGAAGCT
E NCCR bridge Rev	CCATGGATTCTCCCTGTTA
F TCH1 <i>Sac</i> II	GACAAGGCCAAGATTCCGGGAGAATTTAGGGCGG
G Dik3 C65T	GGCCTCAGAAAAGCTTCCACACCTTACTAC
H TCH2 T65C	GGCCTCAGAAAAGCTTCCACACCTTACTAC

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

BKV-NCCRs were amplified by PCR as previously described (Vanchiere et al., 2005b) from the urine of a patient with AIDS and BKV viruria. 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 *Spe*I site was added to each NCCR fragment by a Splicing by Overlap Extension (SOEing) PCR reaction (Horton et al., 1989). Additionally a *Sac*II site was added to the existing S block of each NCCR by site directed mutagenesis. However, the *Sac*II 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 *Spe*I 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 s each of a gradient from 95 °C to 55 °C; annealing at 68 °C for 5 min. The forward *Sac*II primer B, or F for clone TCH1, and the reverse *Spe*I primer A were then added to the above reaction at 4 nM each and PCR amplification was performed using the program: 3 min 30 s at 94 °C; 30 cycles of 45 s 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 *Bam*HI (New England Biolabs, Ipswich, MA) digestion and then recircularized at a concentration of 10 ng/µl using T4 DNA ligase (New England Biolabs). 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 4 or 5 days post-transfection (dpt) by the Hirt protocol (Hirt, 1967). DNA was linearized by digesting with *Bam*HI or *Eco*RI and then digested with *Dpn*I, 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 1× 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 20× SSC using capillary transfer. The membrane was washed for 5 min in 6× 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 BKV *Pvu*II fragment excised from pGEM7-TU (another rearranged variant of BKV) which spans the early region and the NCCR, and was labeled with the Random Primers DNA Labeling System (Invitrogen). The membrane was rehydrated in 6× SSC and incubated for 1 h at 68 °C in prehybridization solution (5× SSC, 1× 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 (5× SSC, 1× Denhardt's solution, 1% SDS) with 0.1 mg/ml denatured sheared salmon sperm DNA and 1× 10⁸ cpm of labeled probe. After hybridization, the membrane was washed with 2× SSC with 0.1% SDS twice for 5 min at room temperature followed by 0.2× 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 2× SSC before being

exposed to film. The Southern blot was quantified using a Typhoon phosphoimager and Imagequant software (GE).

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