REGULATION OF BK VIRUS DNA REPLICATION BY TRANSCRIPTION FACTORS AND NONCODING RNAS

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By

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The undersigned, appointed by the dean of the Graduate School,

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REGULATION OF BK VIRUS DNA REPLICATION BY TRANSCRIPTION FACTORS AND NONCODING RNAS

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A candidate for the degree of

Doctor of Philosophy

And hereby certify that, in their opinion, it is worthy of acceptance.

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In the fall of 2004, I came to United States with one goal to achieve – to become a Ph.D. After my first few weeks' study in University of Missouri-Columbia, I was a little bit worried, because I realized that this goal was not an easy accomplish with my awkward English and limited research experience.

But now, I did it!

I know how many difficulties I had encountered and how much effort I have made to realize this goal. Fortunately, I did not do this alone.

Dr. William Folk, my Ph.D advisor, is always supportive to me. He influences me with his great passion in science and encourages me with his positive altitude. I would have not been able to achieve this without his patient guidance and higher expectation. I appreciate that he has been training me to become an independent scientist and has provided me a lot of opportunities to develop my skills in scientific writing, student mentoring and public speaking, which are all required for a successful scientist.

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This work is dedicated to my family, including my parents, wife and son, who are always proud of me and confident in me. I am greatly indebted to my parents, who spent most of their effort to educate me and promoted me to pursue higher accomplishment, which laid a solid foundation for my Ph.D training. My wife is always very supportive to my work; particularly after we had our baby Bo-yuan, she quitted her job and spent most of her effort to take care of Bo-yuan, so that I could concentrate on my dissertation.

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This is not an end, but just a beginning for a new journey.

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LIST OF ABBREVIATIONS

aa	amino acid
bp	base pair(s)
DMEM	Dulbcco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
kb	kilo base pairs
nt	nucleotide
ori	origin of DNA replication
core-*ori*	core origin of DNA replication
Tag	large T antigen
BKV	BK virus
JCV	JC virus
mPyV	mouse polyomavirus
SV40	Simian virus 40
NFI	Nuclear Factor I
HAT	Histone acetyltransferase
PMSF	phenylmethylsulfonyl fluoride
SDS	sodium dodecyl sulfate

REGULATION OF BK VIRUS DNA REPLICATION BY TRANSCRIPION FACTORS AND NONCODING RNAS

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ABSTRACT

Human polyomavirus BK (BKV) asymptomatically infects 80~90% of people during early childhood, and establishes a life-long persistent infection without causing overt clinical symptoms. High level BKV replication occurs predominantly in urogenital tracts of immune-suppressed patients following renal transplantation and bone marrow transplantation, which cause polyomavirus associated nephropathy (PVAN) and hemorrhagic cystitis, respectively. PVAN has become a leading cause for renal transplantation failures since tacrolimus and mycophenolate mofetil began to be widely used in transplantation patients in 1995. Epidemiological studies indicate that around 30% of kidney transplantation patients are at risk of developing PVAN and 50% of PVAN patients are at risk of transplantation failure.

This dissertation explores the mechanisms by which the genomic noncoding control region (NCCR) regulates BKV DNA replication in cell culture and might be related to the establishment of BKV persistent infection and pathogenesis of PVAN.

These studies indicate that cellular transcription factor NFI interacts with the BKV NCCR and stimulates BKV DNA replication *in vivo* and *in vitro*. Also, the data reveal that isotypes NFIA and NFIB strongly interact with BKV large T antigen (Tag) and NFIC interacts with DNA polymerase-α primase (pol-α primase), suggesting NFI-family transcription factors may stimulate BKV DNA replication through recruitment of Tag and pol-α primase. In contrast, ectopic expression of PCAF/GCN5 histone acetyltransferases inhibit BKV DNA replication. Tag has a site for acetylation by PCAF/GCN5, but inhibition of BKV DNA replication by PCAF/GCN5 is not due to acetylation of Tag, suggesting PCAF/GCN5 target other component(s) of DNA replication machinery. Possible targets include nucleosomes associated with the NCCR and other components of the replication machinery. A search for these targets is proposed, and possible functions of acetylation on BKV Tag are discussed.

BKV DNA does not replicate in murine cells. We and our collaborators have found that this host-restriction of BKV DNA replication involves not only incompatibility of BKV Tag with mouse pol-α primase, but also inhibitory small noncoding RNAs in murine cells, termed srRNAs, that act through BKV NCCR. Specific srRNAs were sequenced and cloned. *In vitro* transcribed srRNAs inhibit BKV replication *in vitro*; and ectopic expression of a specific srRNA strongly inhibits BKV DNA replication *in vivo* in human cells. Surprisingly, srRNAs from human cancer cells stimulate BKV DNA replication *in vitro*, suggesting cell type specific expression of srRNAs has distinct role in regulation of BKV DNA replication. We propose that differential expression of srRNAs may have implication in the viral tropism, establishment of persistent infection and reactivation.

CHAPETER 1

Introduction

BKV Pathogenesis and Polyomavirus associated nephropathy (PVAN)

Human polyomavirus BK (BKV) was firstly isolated in 1971 from the urine of a kidney transplantation patient with ureteral stenosis (84). Epidemiological studies indicate that BKV asymptomatically infects 80~90% of people during early childhood (37, 105, 123, 124, 193). BKV might be transmitted transplacentally(20, 21)or through the fecal-oral route and respiratory tract (18, 19, 89, 110, 164, 211, 229). After initial infection, BKV establishes a life-long persistent infection in kidneys without causing overt clinical symptoms(37, 68, 100). Reactivation of BKV occurs predominantly in urogenital tracts of immune-suppressed patients following renal transplantation and bone marrow transplantation, which cause polyomavirus associated nephropathy (PVAN) and hemorrhagic cystitis, respectively(102, 105).

PVAN has become a leading cause for renal transplantation failures since 1995, when tacrolimus and mycophenolate mofetil (MMF) began to be widely used in transplantation patients (102, 103, 177). Epidemiological studies indicated that around 30% of kidney transplantation patients are at risk of developing PVAN (103, 160) and 50% of PVAN patients are at risk of transplantation failure (42). The other risk factors for PVAN are mismatch of HLA and use of corticosteroids in anti-rejection therapy (12, 103). PVAN is the consequence of extensive BKV replication in kidney allografts, which causes necrotic injury of kidneys tubules (159). The progression of PVAN can be divided into three stages (97, 105, 161). Initially, reactivation and massive replication of BKV in kidney tubular epithelial cells give rise to “inclusions”, the enlarged nuclei filled with viral particles, which

shed from the tubular basement membrane and can be detected as “decoy cells”.In urine samples, these are cytologically useful as surrogate markers for diagnosis of early stage PVAN (Stage A). Extensive shedding and death of tubular epithelial cells cause tubular necrosis and release of pro-inflammatory cytokines and chemokines, which promote the infiltration of leukocytes into the allograft tissue and inflammation, causing “tubulointerstitial nephritis”(Stage B); the final stage in the progression of PVAN is kidney tubular fibrosis and atrophy (Stage C), which is highly associated with allograft loss (64). The current means to control PVAN is to restore immune function by reducing the immune-suppressive regimen, but this increases the risk of allograft rejection(178). Other anti-viral treatments have been attempted, such as cidofovir(8, 76, 125, 220), leflunomide(72, 76, 113, 227), quinolones(6, 81, 172, 181), and immunoglobulin (192), which are not specific and have high toxicity, adding complications to patients.

Polyomavirus replication in acute and persistent infections

BKV has a non-enveloped icosahedral capsid of 40~45nm and a closed circular double-stranded DNA genome(136). It is closely related to two other primate polyomaviruses: SV40 (Simian Virus 40), and JC virus (JCV), the etiological agent for progressive multifocal leukoencephalopathy (PML). BKV DNAs or viral particles have been detected in various organs and tissues(182), including kidney(37, 100), tonsil(89), saliva gland(110), brain(203, 218), lung(218), heart(20), liver(122), lymphocytes(31, 52, 60, 63), but kidney is the primary site for its persistent infection (36, 37, 100). Because the initial acute infection of BKV is usually subclinical, little is known about how it replicates during the initial phase of infection nor how it spreads throughout the body and reaches the kidney to establish a persistent infection. BKV persists at low levels, perhaps as an episome (37, 100), in kidneys at only 0.007+/-0.003 copy/cell, which can increase to 3.4+/-1.8 copies/cell after kidney transplantation and to 7738.9+/-1580.4 copies/cell in allograft kidneys of PVAN patients(179). BKV “viruria” (shedding of virus in urine) occurs

in 0.5~20% immune-competent individuals(102) with titers of ~10^3 copies/ml, and this can increase to >$10^{7\sim8}$ copies/ml in late stage PVAN patients, when "viremia" develops (69, 171). This suggests that during persistent infection BKV replicates at an extremely low level in vivo in immune-competent individuals and the rate of BKV replication increases dramatically in allograft kidneys.

The mechanism(s) that maintain BKV low-level replication in kidney during persistent infection are likely to include inhibition of viral replication by immune factors (42) and/or other inhibitory cellular factor(s). Studies have shown that interferon-gamma inhibits the replication of BKV *in vitro* (3); antibodies against BKV capsid protein VP1 have been detected in the serum of people with latent BKV infection and PVAN patients (98, 124, 162); and cell-mediated immunity is crucial for the control of BKV (17, 70, 237). However, the immune system does not completely eradicate BKV in the human host, indicating BKV can evade immune surveillance. A recent study showed that a miRNA expressed by BKV and JCV targets the ULBP3, a stress-induced ligand expressed on surface of infected cells, to down-regulate both the native and adaptive immune response against viral infection(13). Also, high-throughput assays of cellular gene expression in response to BKV infection *in vitro* indicated that several genes responsible for immune control are regulated upon BKV infection (2, 90). But their consequences for viral replication has not been determined. Replication of BKV might be restricted to a low-level, to escape immune surveillance, perhaps through control by cis-elements in the viral noncoding control region (NCCR) or through modulation of large T antigen (Tag) expression or activity by posttranslational modifications. This is partially supported by the observations that replication of archetype BKV is very inefficient (27, 69, 88, 189). More details of how the NCCR and modification of T-Ag might restrict BKV replication will be discussed below.

Also important is why and how BKV replication is reactivated in PVAN. Evidence suggests that immune-suppression is necessary but not sufficient for PVAN to occur. PVAN is only highly associated with kidney transplantation patients and rarely occurs in autologous kidneys of patients with other immunocompromsed conditions (78, 157). BKV reactivates in some patients with bone marrow transplantation, but this causes hemorrhagic cystitis, not PVAN (14, 102, 105). Only sporadic cases of HIV+ individuals have been reported to have symptoms of PVAN(158, 200) and PVAN rarely occurs in recipients of other organ transplants (liver, heart, pancreas, etc.) even though they receive similar and in some cases, even stronger immune-suppression regimens (105). And PVAN is extremely rare in patients with other immune-compromised conditions, such as systemic lupus erythematosus (SLE)(210) and leukemia(203). These suggest that in addition to immune suppression, other factors related to certain conditions of allograft kidney are particularly important for reactivation of BKV and the progression of PVAN. This might be related to allograft ischemia/reperfusion injury and regeneration, inflammatory response, which in turn, lead to activation of DNA damage/repair and/or mitogenic pathways utilized by polyomaviruses (10, 78, 102, 104).

JCV, another human polyomavirus with 80% of homology to BKV genome sequence, also persists in kidneys of most people and reactivates predominantly in AIDS patients, however, and this viral reactivation causes Progressive Multifocal Leukoencephalopathy (PML). Although JC virus replication has been detected in some renal transplantation patients, the incidence of JCV infection in kidney transplantation is very low and is generally not associated with disease progression of PVAN (145). This suggests that BKV has unique properties related to certain treatment conditions used in renal transplantation that account for its reactivation specifically in kidney allografts and the pathogenesis of PVAN.

Due to the lack of animal model for PVAN, little is known about the how BKV replication is regulated during acute and persistent infection *in vivo* in human population. However, replication of mouse polyomavirus (mPyV) during acute and persistent infection has been studied extensively in mice. As with other polyomaviruses, mPyV infection has an acute phase, a clearance phase and a persistent phase (66). The acute phase is usually within 6 days postinfection (dpi) in the respiratory tracts, when viral replication reaches maximum levels, and the infection spreads systematically from lungs to different organs such as kidneys, livers and spleens; the viral load reaches up to >1000 copies/cell in lungs and ~10 copies/cell in kidneys. During the clearance phase, 6~12 dpi, virus titers drop significantly throughout all the organs, to undetectable levels except for kidneys and lungs, where virus can be detected at 1 copy/cell in kidneys and 10 copies/cell in lungs. Finally, in the persistent phase, low level infection is established from 22 dpi to 84 dpi where viral DNA is present at ~1 copies/cell in lungs and <1 copies/cell in kidneys (65, 66). During the long-term persistent infection, viral DNA exists as a free supercoiled molecule in lungs and kidneys, without detectable integration into the host genome(66). Reactivation of mPyV replication can be induced in kidneys, when kidneys are injured by chemicals (glycerol, cisplatin) or ischemia insult (clamping of kidney arteries), but not by immune-suppression (methotrexate)(10, 186).

The mPyV noncoding control region (NCCR) controls viral DNA replication during both acute and persistent infection(184-186). Mutation of specific cis-elements, particularly transcription factor binding sites, in the enhancer region changes organ-specific replication of mPyV during acute infection in vivo (184, 185). The enhancer also regulates persistent infection (186): mPyV with a mutated enhancer that strongly stimulated viral replication in acute infection failed to establish a long-term persistent infection(186), suggesting the NCCR regulates viral replication in different ways during acute and persistent infection. This is reminiscent of what has been observed with BKV with

rearranged enhancers, that replicate efficiently in cell culture but are distinct from the archetype form that persists naturally in kidneys (27, 88, 163).

These observations suggest that the NCCR of BKV has an important role in the reactivation of replication and the ensuing pathogenesis of PVAN. The major goal of this dissertation research is to study the mechanism(s) by which the BKV NCCR modulates viral DNA replication, and to relate these mechanisms to BKV persistent infection and reactivation in PVAN .

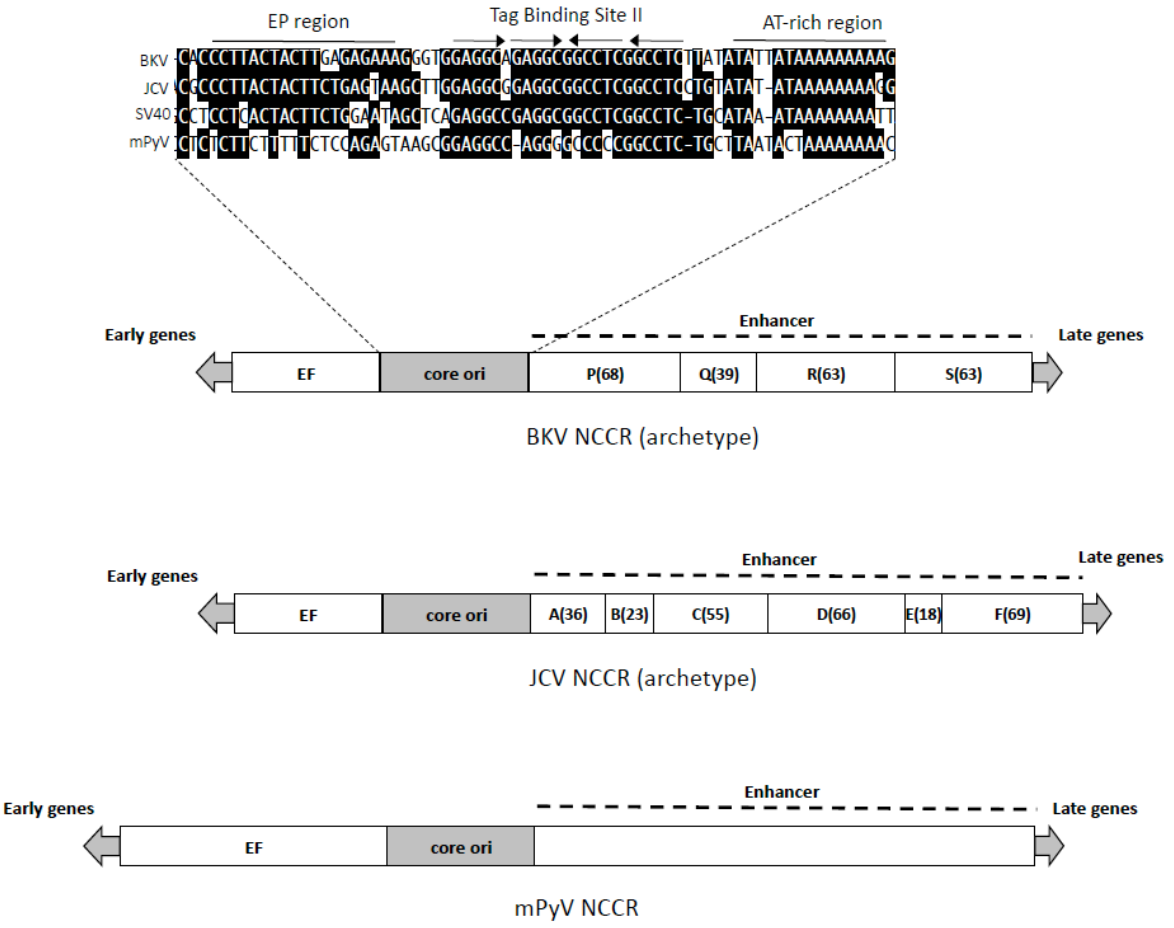
The viral non-coding control region (NCCR) and its regulatory role in polyomavirus transcription and DNA replication

The NCCR contains the origin of DNA replication and promoter/enhancer elements for regulation of viral early/late gene expression and DNA replication. (38, 149, 225). NCCRs of BKV, JCV, SV40 and mPyV have similar organization and can be divided into three regions: the core origin, early core origin flanking sequence (EF) and late core origin flanking sequence (enhancer)(Figure 1.1).

The core origin is the minimal sequence required for polymavirus DNA replication *in vitro*. Sequences of SV40(50, 51, 54, 130), BKV(58), JCV(47, 80), mPyV(45, 116, 154, 176) core origins have been well defined. Alignment of these core origin sequence sequences (Figure 1.1) indicates that they consist of three essential elements: a large T-antigen (Tag) binding site II (a palindrome of four “G(A/G)GGC” pentanucleotide repeats), an AT-rich region and an early imperfect palindrome (EP) region. Detailed biochemical analysis of the SV40 origin suggested that DNA replication initiates upon Tag binding and formation of double hexamers at the “Tag binding site II” in the presence of ATP (24, 141, 166, 219), followed by melting of duplex DNA in the “EP region” and bending/untwisting

Figure 1.1 Organization of NCCRs of BKV, JCV and mPyV. BKV, JCV and mPyV NCCRs are divided into three regions: core origin (core ori), early flanking sequence (EF) and Enhancer. Alignment of core ori sequences from BKV, JCV, SV40 and mPyV is shown. Positions of Early palindrome region (EP region), Tag Binding site II and AT-rich region of SV40 core ori are marked above the aligned sequences. The configurations of archetype BKV and JCV NCCRs are illustrated; numbers in brackets are numbers of nucleotides in each block.

Figure 1.1


EP region
Tag Binding Site II
AT-rich region
BKV
JCV
SV40
mPyV
Early genes
Enhancer
Late genes
EF
core ori
P(68)
Q(39)
R(63)
S(63)
BKV NCCR (archetype)
Early genes
Enhancer
Late genes
EF
core ori
A(36)
B(23)
C(55)
D(66)
E(18)
F(69)
JCV NCCR (archetype)
Early genes
Enhancer
Late genes
EF
core ori
mPyV NCCR

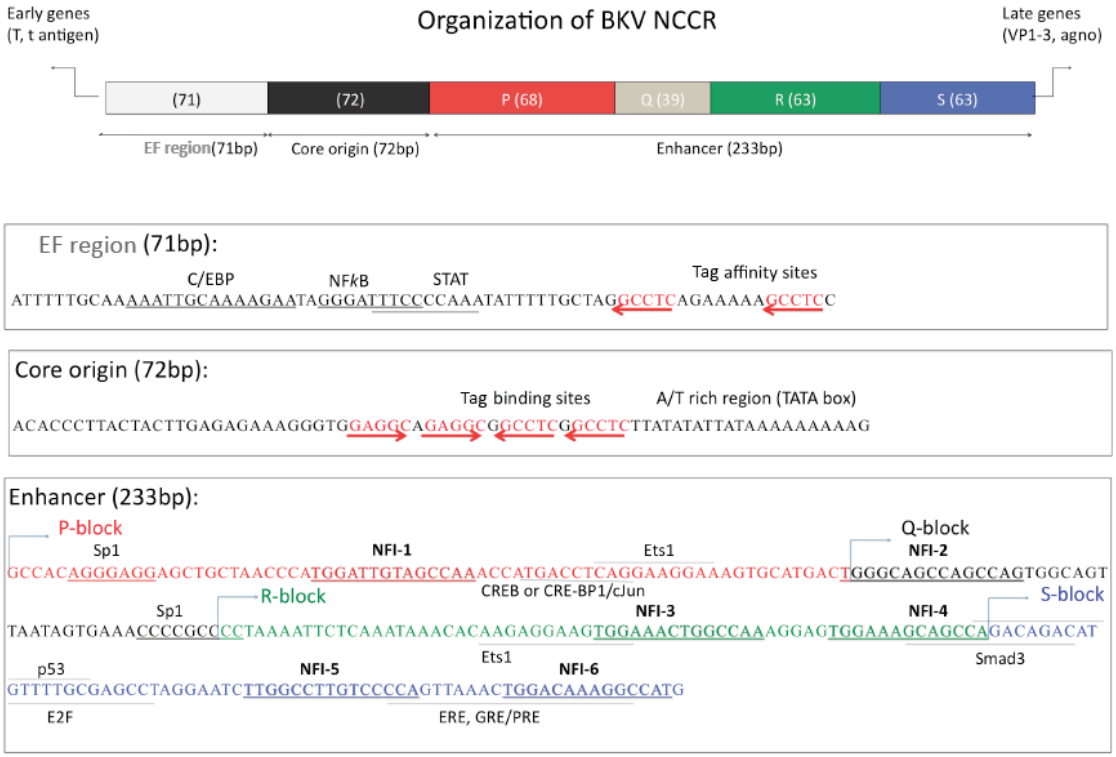
of DNA in the" AT-rich region" (25, 51, 101, 165). Tag double hexamers unwind the DNA duplex in opposite directions with the Tag helicase/ATPase activity in the presence of ATP/Mg2+ (49, 199, 228). Other components of the DNA replication initiation complex, including RPA, Topoisomerase I and DNA polyomerase -α primase (pol-α primase) are recruited to the core origin to initiate viral DNA replication.

While the core origin is essential for BKV DNA replication, the EF and enhancer control viral gene transcription and regulate DNA replication (149, 225). Many transcription factors binding sites have been identified in the BKV core origin flanking sequences (Figure 1.2). In the EF region, binding sites for NF*k*B(86), C/EBP(86), GM-CSF(149, 180) have been identified, and the NF*k*B site was shown to stimulate BKV early gene expression synergistically with C/EBP(180). Although the function of the GM-CSF site is not clear, it has been reported to be highly mutated (~90%) in PVAN samples suggesting its importance for BKV reactivation in PVAN(180). In the BKV enhancer region, binding sites for NFI(29, 30, 59, 91, 137), Sp1(29, 59, 138), GRE/PRE (glucocoticoid/progesterone responsive element) and ERE (estrogen responsive element) (150), NF-AT(112), p53(197), AP1(30, 138, 139), Smad3(1) were identified and shown to regulate early/late gene expression. Many more putative binding sites of transcription factors, including Ets-family transcription factors (PEA-3, Ets-1 and Spi-1/PU.1), CREB, AP-2, NF*k*B were predicted in BKV enhancer region, however, their roles in BKV transcription are unclear (149).

Transcription factors not only regulate gene transcription, but also modulate DNA replication (reviewed in 55, 56, 57, 156). Mechanisms by which transcription factors stimulate polyomavirus DNA include: 1) recruitment of replication factors to the origin to facilitate assembly of the replication complex: AP-1 and VP16 stimulated mPyV replication by recruitment of Tag and RPA (99, 109); 2) remodeling of chromatin configuration into an active form: NFI stimulates SV40 DNA replication by preventing the

Figure 1.2 Organization of BKV NCCR and transcription factor binding sites in core origin flanking sequences. Tag binding and affinity sites are marked with red arrows. Transcription factor binding sites are marked with black underlines. Six NFI sites are numbered from NFI-1 to -6.

Figure 1.2


Organization of BKV NCCR
Early genes
(T, t antigen)
Late genes
(VP1-3, agno)
(71)
(72)
P (68)
Q (39)
R (63)
S (63)
EF region(71bp)
Core origin (72bp)
Enhancer (233bp)
EF region (71bp):
C/EBP
NFkB
STAT
Tag affinity sites
ATTTTTGCAAAAATTGCAAAAGAATAGGGATTTCCCCAAATATTTTTGCTAGGCCTCAGAAAAAGCCTCC
Core origin (72bp):
Tag binding sites
A/T rich region (TATA box)
ACACCCTTACTACTTGAGAGAAAGGGTGGAGGCAGAGGCGGCCTCGGCCTCTTATATATTATAAAAAAAAAG
Enhancer (233bp):
P-block
Sp1
NFI-1
Ets1
Q-block
NFI-2
GCCACAGGGAGGAGCTGCTAACCCATGGATTGTAGCCAAACCATGACCTCAGGAAGGAAAGTGCATGACTGGGCAGCCAGCCAGTGGCAGT
CREB or CRE-BP1/cJun
R-block
Sp1
NFI-3
NFI-4
S-block
TAATAGTGAAACCCCCGCCCCTAAAATTCTCAAATAAACACAAGAGGAAGTGGAAACTGGCCAAAGGAGTGGAAAGCAGCCAGACAGACAT
Ets1
Smad3
p53
NFI-5
NFI-6
GTTTTGCGAGCCTAGGAATCTTGGCCTTGTCCCCAGTTAAACTGGACAAAGGCCATG
E2F
ERE, GRE/PRE

formation of repressive chromatin(35) and relievies repressive chromatin through interaction with histone H3(153); 3) induction of DNA structure changes in the origin to promote initiation: Sp1 stabilizes the bent structure of SV40 origin DNA caused by Tag during the initiation(128, 208, 226); 4) facilitating the intracellular localization to promote DNA replication: Runx1 recruits mPyV origin to replication factories on the nuclear matrix by In addition to stimulation(33, 155). Transcription factors may also inhibit viral DNA replication, for instance: Oct-1 inhibits SV40 DNA replication when bound to AT-rich region in core origin(120); p53 inhibits SV40 DNA replication *in vitro* and *in vivo* at the initiation stage, perhaps by interfering with interaction of Tag with RPA or origin, or inhibiting helicase activity of Tag (67, 147, 205, 221).

The requirement for core origin flanking sequences for viral DNA replication is different for different polyomaviruses. The core origin flanking sequences of mPyV is required for DNA replication *in vitro* and *in vivo* (48, 152, 176); while those of SV40 are not absolutely required *in vitro*, they stimulate SV40 replication by 10~100 fold *in vivo* and *in vitro* (95, 96). One study showed that BKV core origin flanking sequences do not enhance BKV DNA replication in CV-1 and HeLa cells transiently expressing BKV Tag (58), but the BKV enhancer stimulates SV40 core origin replication by SV40 Tag in COS-1 cells (58). The importance of the core origin flanking sequences for BKV DNA replication in human kidney tubular epithelial cells is not clear. Another study indicated that in addition to core origin, a 21bp fragment of P block flanking sequence is required for BKV replication in COS-1 cells (53). These suggest that the requirement of core origin flanking sequences for polyomavirus DNA replication might be cell-type dependent and the specific composition of *trans*-acting elements in different types of cells determines how core origin flanking sequences regulate polyomavirus DNA replication.

The NCCR sequences of BKV and JCV isolated from clinical samples and selected for replication in cell culture display great heterogeneity(151). It is now believed that all

variants are originated from an archetype NCCR (at-NCCR) through rearrangement of enhancer sequence by deletion, duplication and insertion during reactivation or passage of virus in cell culture. Compared with enhancer, core origin and EF region are relatively stable.

The archetype BKV and JCV viruses have genomes that persist latently in kidney and are transmitted among the population. For both BKV and JCV, the archetype enhancer has a linear array of several blocks of sequences arbitrarily designated as P_{68}-Q_{23}-R_{63}-S_{63} for BKV(149), and A_{36}-B_{23}-C_{55}-D_{66}-E_{18}-F_{69} for JCV(11, 79, 232). The most widely studied archetype strain of BKV is the WW strain (ww-NCCR) directly cloned from urine without passage in cell culture(32, 146, 188). Other archetype strains with a few single nucleotide changes have been reported, such as WWT(209) and Dik(213). Phylogenetic analysis of these archetype sequences indicates that these single-nucleotide changes are due to random mutations accumulated during the natural evolution of the virus and are not related to viral pathogenesis (233).

It has been suggested that BKVs with rearranged NCCRs (rr-NCCRs) are generated through recombination and gain growth advantage through positive selection during in vivo replication or upon passage of virus in cell culture, so they replicate more efficiently than virus with at-NCCR. In support of this, quasispecies of BKV with rr-NCCRs were detected in kidney transplant patients and most of them replicate faster than the archetype NCCR (at-NCCR) in cell culture (88, 163). Isolates of several BKV rr-NCCRs from HIV+ individuals also displayed similar enhanced replication activity (27). And rr-NCCRs with the same configuration were repeatedly detected in a single patient during the course of PVAN progression (167), suggesting they kept replicating stably at high-level in PVAN patients. But how the rearrangement of BKV NCCR is related to the pathogenesis of PVAN is still not clear. The extensive replication of archetype BKV upon reactivation gives rise to BKVs with rr-NCCRs, some of which may replicate in cells that

are usually non-permissive for archetype virus.In support of this, it was shown that rr-NCCRs were more prevalent in PVAN patient than patients with only viruria(194); and a recent longitudinal study of BKV genotype in kidney transplantation patients shows a discordance of appearance of rr-NCCR in plasma and ww-NCCR in urine at the same time, suggesting the BKVs with rr-NCCR replicate preferentially in different compartments with ww-NCCR(88). Similarly, JCV archetype also persists in kidney, but the NCCRs of viruses isolated from brain and CSF of PML patients are rearranged (87). And rearranged JCV replicates efficiently in cultured glial cells and kidney cells, which are not permissive for archetype JCV(47, 87). It is hypothesized that the at-NCCR with lower replication efficiency is particularly adapted for persistent infection because its low level of replication protects the latent virus from immune surveillance (88).

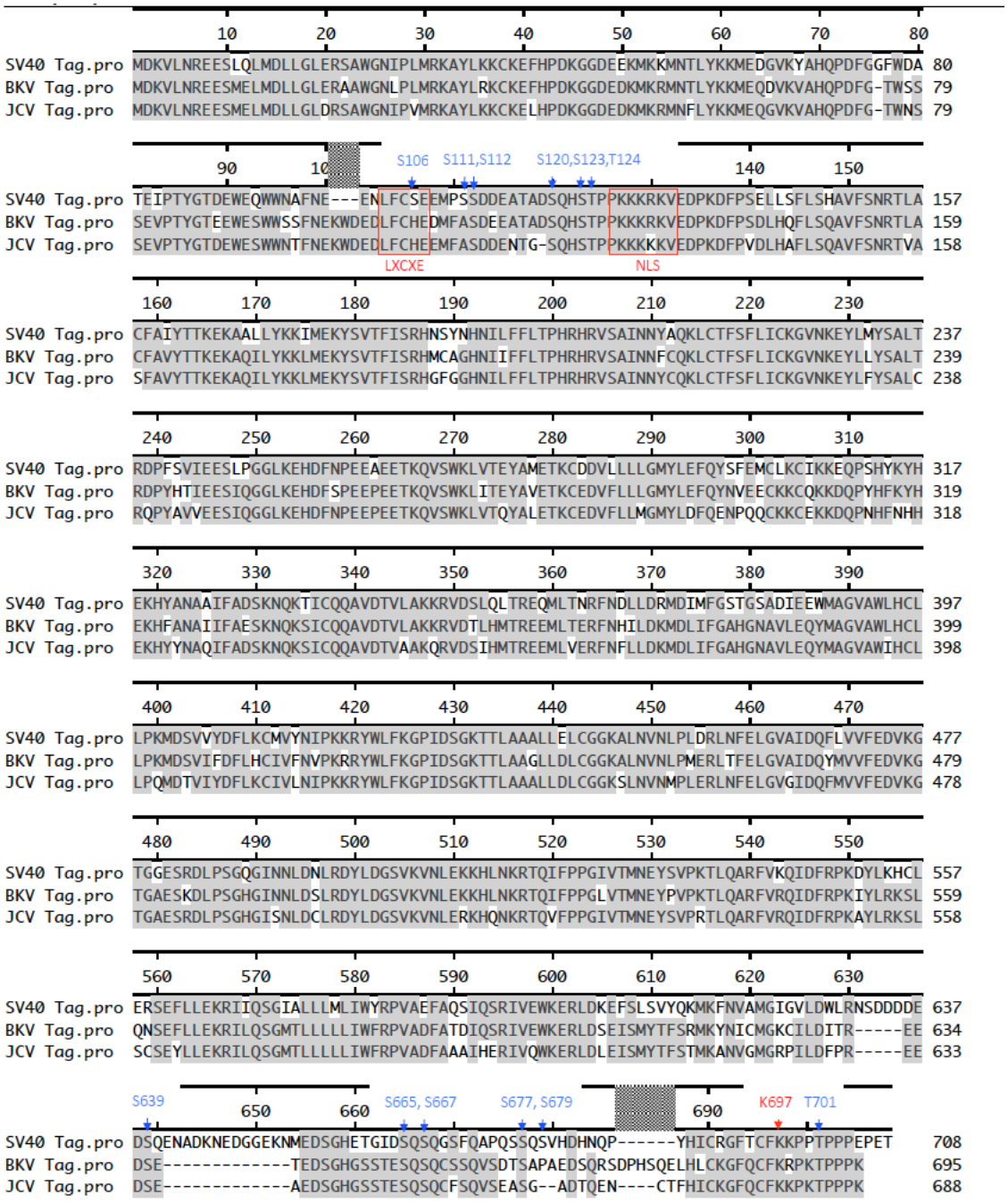
Large T-antigen (Tag)

Replication of polyomaviruses requires the coordination of Tag with series of cellular proteins involved in DNA replication, transcription, cell cycle control, cell transformation and DNA damage response. SV40 Tag's structural and functional domains have been well characterized (reviewed in references 4, 5, 23, 34, 73-75). Tags of BKV, JCV and SV40 have 75% of homology; and mouse polyomavirus (mPyV) shares 45~55% homology with SV40 Tag (169). So by alignment of their protein sequences, we may infer the structural and functional domains of BKV and JCV Tags from what's known about SV40 Tag (Figure 1.3A). In spite of great similarities, Tags of these closely related polyomaviruses have subtle but important differences in structure and function, which are reviewed below.

SV40 Tag is a 90~100kD polypeptide of 708 amino acids and consists of four major independent structural domains determined from their proteolysis pattern and distinct

Figure 1.3 Structure and functional domains of Tags. (A) Alignment of amino acid sequences of SV40, BKV and JCV Tags. “LxCxE” motifs and NLSs (nuclear localization signals) are marked with red line box. Phosphorylation sites of SV40 Tag are marked with blue arrows. Acetylation site of SV40 Tag is marked with red arrow.

Figure 1.3A


SV40 Tag.pro MDKVLNREESLQLMDLLGLERSAWGNIPLMRKAYLKKCKEFHPDKGGDEEKMKKMNTLYKKMEDGVKYAHQPDFGGFWDA 80
BKV Tag.pro MDKVLNREESMELMDLLGLERAAWGNLPLMRKAYLRKCKEFHPDKGGDEDKMKRMNTLYKKMEQDVKVAHQPDFG-TWSS 79
JCV Tag.pro MDKVLNREESMELMDLLGLDRSAWGNIPVMRKAYLKKCKELHPDKGGDEDKMKRMNFLYKKMEQGVKVAHQPDFG-TWNS 79
S106
S111,S112
S120,S123,T124
SV40 Tag.pro TEIPTYGTDEWEQWWNAFNE---ENLFCSEEMPSSDDEATADSQHSTPPKKKRKVEDPKDFPSELLSFLSHAVFSNRTLA 157
BKV Tag.pro SEVPTYGTEEWESWWSSFNEKWDEDLFCHEDMFASDEEATADSQHSTPPKKKRKVEDPKDFPSDLHQFLSQAVFSNRTLA 159
JCV Tag.pro SEVPTYGTDEWESWWNTFNEKWDEDLFCHEEMFASDDENTG-SQHSTPPKKKKKVEDPKDFPVDLHAFLSQAVFSNRTVA 158
LXCXE
NLS
SV40 Tag.pro CFAIYTTKEKAALLYKKIMEKYSVTFISRHNSYNHNILFFLTPHRHRVSAINNYAQKLCTFSFLICKGVNKEYLMYSALT 237
BKV Tag.pro CFAVYTTKEKAQILYKKLMEKYSVTFISRHMCAGHNIFFLTPHRHRVSAINNFCQKLCTFSFLICKGVNKEYLLYSALT 239
JCV Tag.pro SFAVYTTKEKAQILYKKLMEKYSVTFISRHGFGGHNILFFLTPHRHRVSAINNYCQKLCTFSFLICKGVNKEYLFYSALC 238
SV40 Tag.pro RDPFSVIEESLPGGLKEHDFNPEEAEETKQVSWKLVTEYAMETKCDDVLLLGMYLEFQYSFEMCLKCIKKEQPSHKYH 317
BKV Tag.pro RDPYHTIEESIQGGLKEHDFSPEEPEETKQVSWKLITEYAVETKCEDVFLLGMYLEFQYNVEECKKCQKKDQPYHFKYH 319
JCV Tag.pro RQPYAVVEESIQGGLKEHDFNPEEPEETKQVSWKLVTQYALETKCEDVFLLMGMYLDFQENPQQCKKCEKKDQPNHFNHH 318
SV40 Tag.pro EKHYANAAIFADSKNQKTICQQAVDTVLAKKRVDSLQLTREQMLTNRFNDLLDRMDIMFGSTGSADIEEWMAGVAWLHCL 397
BKV Tag.pro EKHFANAIIFAESKNQKSICQQAVDTVLAKKRVDTLHMTREEMLTERFNHILDKMDLIFGAHGNAVLEQYMAGVAWLHCL 399
JCV Tag.pro EKHYYNAQIFADSKNQKSICQQAVDTVAAKQRVDSIHMTREEMLVERFNFLLDKMDLIFGAHGNAVLEQYMAGVAWIHCL 398
SV40 Tag.pro LPKMDSVVYDFLKCMVYNIPKKRYWLFKGPIDSGKTTLAAALLELCGGKALNVNLPLDRLNFELGVAIDQFLVVFEDVKG 477
BKV Tag.pro LPKMDSVIFDFLHCIVFNVPKRRYWLFKGPIDSGKTTLAAGLLDLCGGKALNVNLPMERLTFELGVAIDQYMVVFEDVKG 479
JCV Tag.pro LPQMDTVIYDFLKCIVLNIPKKRYWLFKGPIDSGKTTLAAALLDLCGGKSLNVNMPLERLNFELGVGIDQFMVVFEDVKG 478
SV40 Tag.pro TGGESRDLPSGQGINNLDNLRDYLDGSVKVNLEKKHLNKRTQIFPPGIVTMNEYSVPKTLQARFVKQIDFRPKDYLKHCL 557
BKV Tag.pro TGAESKDLPSGHGINNLDSLRDYLDGSVKVNLEKKHLNKRTQIFPPGLVTMNEYPVPKTLQARFVRQIDFRPKIYLRKSL 559
JCV Tag.pro TGAESRDLPSGHGISNLDCLRDYLDGSVKVNLERKHQNKRTQVFPPGIVTMNEYSVPRTLQARFVRQIDFRPKAYLRKSL 558
SV40 Tag.pro ERSEFLLEKRIIQSGIALLLMLIWYRPVAEFAQSIQSRIVEWKERLDKEFSLSVYQKMKFNVAMGIGVLDWLRNSDDDDE 637
BKV Tag.pro QNSEFLLEKRILQSGMTLLLLLIWFRPVADFATDIQSRIVEWKERLDSEISMYTFSRMKYNICMGKCILDITR-----EE 634
JCV Tag.pro SCSEYLLEKRILQSGMTLLLLLIWFRPVADFAAAIHERIVQWKERLDLEISMYTFSTMKANVGMGRPILDFPR-----EE 633
S639
S665, S667
S677, S679
K697
T701
SV40 Tag.pro DSQENADKNEDGGEKNMEDSGHETGIDSQSQGSFQAPQSSQSVHDHNQP------YHICRGFTCFKKPPTPPPEPET 708
BKV Tag.pro DSE-------------TEDSGHGSSTESQSQCSSQVSDTSAPAEDSQRSDPHSQELHLCKGFQCFKRPKTPPPK 695
JCV Tag.pro DSE-------------AEDSGHGSSTESQSQCFSQVSEASG--ADTQEN----CTFHICKGFQCFKKPKTPPPK 688

functions (reviewed in references 73, 74, 169) (Figure 1.3B). The N-terminal J domain (1-82aa) is a homolog of cellular DnaJ chaperon that interacts with Hsc70 to promote cellular transformation by disrupting Rb-E2F complex (204, 206, 207, 235); in addition, J domain also facilitates DNA replication in vivo, perhaps by promoting the DNA binding and hexamer assembly (28, 222). The origin-binding domain (*Ori*-binding) (131-259aa) is responsible for binding to the origin of replication and contains the contact interface between two Tag hexamers(9, 107, 143). The Helicase/ATPase domain (251-627aa) has intrinsic helicase and ATPase activity, with which Tag double-hexamers unwind duplex DNA by hydrolysis of ATP(83, 129). The Zn Finger motif (302-320aa) within the Helicase/ATPase domain is required for Tag hexamer assembly (133) and sequence-specific origin binding(9, 107). The structures of J domain, *Ori*-binding domain and helicase/ATPase domain have been resolved (121, 129, 134). But the structure and function of the C-terminal host range domain (HR) (628-708aa) is not clear. Replication of mutant SV40 with deletions of the HR domain is defective in CV-1 cells (170, 216, 217) and temperature sensitive in BSC cells (40, 170), which can be rescued by providing the HR domain *in trans* (173, 217). It also has a similar helper function (*hf*) to allow adenovirus to grow in monkey cells when provided *in trans* (39, 92, 117). HR domain is not absolutely required for DNA replication, but might function in regulating viral late gene expression or capsid assembly(201, 202, 238). The linker region between J domain and DNA binding domain is a relatively unstructured region, which contains the docking sites for many cellular proteins, including the pRb-related proteins (the “LXCXE” motif)(71, 117), Cul7(7), Bub1(43), Fbw7(224), IRS1(77) as well as a NLS (nuclear localization signal)(114, 115, 126, 127).

Tags of BKV (695aa) and JCV (688aa) are similar in size compared with SV40 Tag and have very similar organization of their functional domains. But the amino acid sequence of mPyV Tag (785aa) is longer than the three Tags of other primate polyomaviruses (85). The J domain, *Ori*-binding domain, Helicase/ATPase domain of Tags from SV40, BKV

Figure 1.3 (B) Organization of functional domains of SV40 Tag. SV40 Tag (708aa) has four major functional domains: J domain (aa1-82); origin binding domain (Ori-Binding) (aa131-259); Helicase/ATPase domain (aa251-627); host range (HR) domain (aa682-708). Zn-finger and ATPase domain are located in aa302-320 and aa418-627 within Helicase/ATPase domain. Nuclear localization signal (NLS) is located at aa126-132. There is a linker sequence between Helicase/ATPase and HR domain, which is less conserved in SV40, BKV and JCV Tags. Domains within SV40 Tag shown to interact with cellular proteins including HSC70, pRb family proteins, p53, Cul7, Bub1, Fbw7, IRS1, pol-α primase, RPA, Topoisomerase I, Nbs1, ATM/ATR, are illustrated below. (C) Organization of functional domains of mPyV Tag. mPyV Tag (785aa) has three major function domains: J domain (aa1-79); Ori-Binding (aa282-398); Helicase/ATPase domain (aa398-785). Two NLSs are located at aa189-195 and aa280-286. Zn-finger and ATPase domains within Helicase/ATPase domain are located at aa452-472 and aa565-785.

Figure 1.3 B

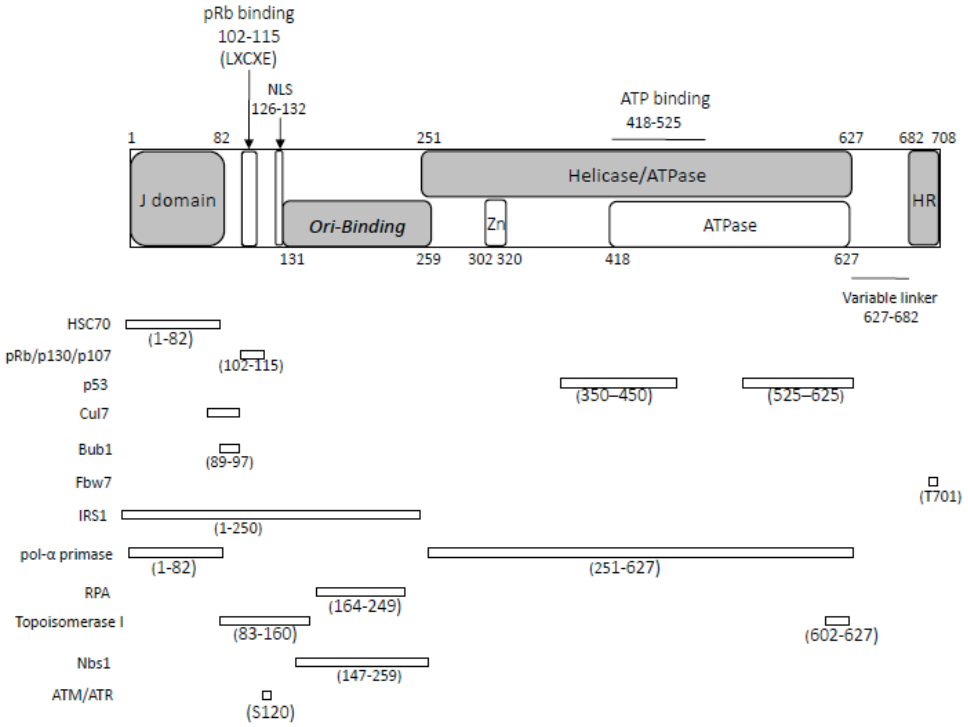
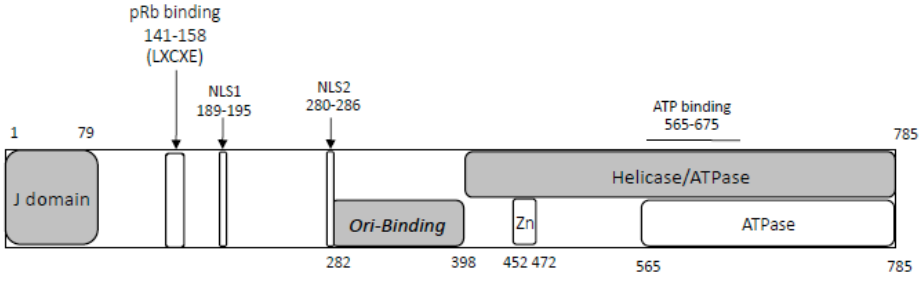


Figure 1.3 C



and JCV are highly conserved, while their C-terminal end regions with a variable linker and the HR domain are quite different, particularly between SV40 and two human polyomaviruses, BKV and JCV (Figure 1.3A). Tag of mPyV also has a J domain(15), an Ori-binding domain(212) and a Helicase/ATPase domain(26), but lacks the C-terminal region of SV40, BKV and JCV Tags (Figure 1.3C). Remarkably, the unstructured linker region of mPyV Tag between J domain and Ori-binding domain is ~150aa longer than the similar linker region in SV40 Tag (Figure 1.3B); and it has two NLSs, the first one (NLS1) is unique to mPyV, and the other one (NLS2) is conserved with the SV40 NLS (108, 183). Why mPyV has two NLSs is not clear. The "LxCxE" motif is also present in mPyV Tag's linker region(168), but whether other proteins docking to the linker region of SV40 Tag also bind to mPyV remains unknown.

Tag carries out its functions through interaction with many cellular proteins (Figure 1.3B), including: 1) proteins of DNA replication machinery, including pol-α primase (41, 61, 62), RPA(144, 223), Topoisomerase I (82, 118, 187, 198) to facilitate initiation of DNA replication; 2) proteins responsible for cell cycle control, including pRb, p53 (119, 131, 132, 190), Cul7(7), Bub1(43), Fbw7(224), IRS1(77) to promote S-phase entry and transformation; 3) components of DNA damage response, including ATM/ATR(44, 196, 236), Nbs1(230) to bypass and co-opt this machinery; 4)transcription factors including p53 (147, 205, 221), c-Jun(16, 94, 109), Sp1(111), AP2(148) and components of RNApol-II(RNA polymerase II) transcriptional initiation complex, including TBP(TATA box-binding protein)(46, 93, 111, 140),TAFs(TBP asoosciated factors)(46) and RNA pol-II(111), to regulate viral/cellular gene transcription and DNA replication; 5) histone acetyltransferases (HATs), including CBP/p300(22, 174) and PCAF/GCN5(231), to acetylate Tag for unknown function. These proteins may also interact with BKV Tag for similar functions. In support of this, Rb and p53 have been shown to interact with BKV Tag. Whether BKV Tag interacts with other proteins has not been shown.

Another way for Tag to regulate its activity is through post-translational modifications. Several kinds of post-transplantation modifications of SV40 Tag have been reported (74), including phosphorylation, amino-terminal and specific lysine acetylations, O-glycosylation, acylation, adenylylation, poly(ADP-ribosly)-ation. Phosphorylation of SV40 Tag has been studied extensively(reivewed in references 73, 74, 175).

SV40 Tag is phosphorylated at serines and threonines clustered in two regions, one in the N-terminus linker region between J domain and *Ori*-binding domain (Ser106, Ser111, Ser112, Ser120, Ser123, Thr124), and the other in the C-terminus region (Ser639, Ser 665, Ser667, Ser677, Ser679, Thr701)(Figure1.3A). Mutations of Ser120, Ser 123, Thr124 and Ser679 have significant but distinct effects on viral DNA replication. Phosphorylation of Ser 120 and Ser 123 inhibits viral DNA replication *in vitro*, but mutation of Ser120 and Ser123 to Ala greatly reduce viral replication *in vivo*(191), suggesting the inhibition of viral DNA replication by phosphorylation on these sites might be essential for viral infection cycle *in vivo*. It was found recently that Ser120 is phosphorylated by ATM kinase upon SV40 infection, suggesting this or other phosphorylation sites might be important for the virus to co-opt the DNA damage response triggered by viral infection (195). In contrast, phosphorylation of Thr124 by cdc3 kinase greatly enhanced Tag's replication activity *in vitro*(106, 142); and mutations of Thr124 to Ala completely abolished SV40 Tag's origin binding and DNA replication activity (191). Importantly, the Ser120, Ser123 and Thr124 sites are conserved in BKV and JCV Tags, suggesting they are essential for viral replication. Phosphorylation of Ser679 down-regulated DNA replication *in vitro* and SV40 with mutation at Ser679 replicated better than wildtype virus *in vivo*(191). However, Ser679 is not conserved in BKV and JCV Tag (Figure 1.3A), suggesting that BKV and JCV Tag might have a higher replication activity than SV40. It has recently been shown that phosphorylated Thr701 interacts with tumor suppressor Fwb7 to prevent degradation of cyclinE, perhaps, to

promote cell cycle progression and cell transformation(224). The Thr701 is conserved in BKV and JCV Tag (Figure 1.3A). The functions of other phosphorylation sites remain unknown.

SV40 Tag can be acetylated by histone acetyltransferases (HATs) CBP/p300 at the K697 residue in the C-terminus Host Range domain through formation of a ternary complex mediated by p53(22, 174). However, mutation of K697 does not affect the host range activity of Tag's C-terminal region (173), suggesting the acetylation has another modulatory function. Another group of HATs, PCAF/GCN5, but not CBP/p300, acetylates mouse polyomavirus Tag and stimulates mouse polyomavirus DNA replication, when tethered to the origin (231), suggesting acetylation of Tag might stimulate polyomavirus DNA replication. Interestingly, the acetylation site and its surrounding residues of SV40 Tag are highly conserved among SV40, BKV and JCV (Figure 1.3A), suggesting the acetylation might play an important role in viral replication and/or pathogenesis. Whether CBP/p300 or PCAF/GCN5 acetylates BKV Tag has not been reported.

Objectives

The primary goal of this thesis research is to determine how cellular factors binding to BKV NCCR regulate BKV DNA replication and to relate this to the BKV reactivation in PVAN. This research started from a collaborative effort with Dr. Heinz-Peter Nasheuer and Dr. Michael Imperiale's laboratories to study the mechanism(s) responsible for restriction of BKV DNA replication in murine cells. Several discoveries have been made, three of which have already been published (135, 214, 215), and others will be submitted for publication after completion of this dissertation.

Previous studies in the Folk lab have established that transcription factors stimulate mPyV DNA replication through interaction with viral NCCR(94, 234). We explored how

similar mechanism regulates BKV DNA replication; and most importantly, how it contributes to BKV reactivation in PVAN.

Chapter 2 describes the stimulation of BKV DNA replication by NCCR and identification of the NFI-family transcription factors that stimulate BKV DNA replication through binding to NCCR, when Tag and pol-α primase are limiting. Interactions of NFIs with Tag and pol-α primase have been detected, suggesting NFIs stimulate BKV DNA replication through recruitment of Tag and pol-α primase to replication origin.

It has been shown that HATs PCAF/GCN5 stimulate mPyV DNA replication, perhaphs through modification of Tag(231). Whether HATs modulate BKV DNA replication has been investigated in this dissertation research. As shown in Chapter 3, PCAF/GCN5 HATs acetylate BKV Tag at a K687 residue in the C-terminal HR domain; but PCAF/GCN5 modulate BKV and mPyV DNA replication in distinct manners. Point mutations of BKV and mPyV Tags indicate that acetylation of Tags appears to be not directly involved in DNA replication of either mPyV or BKV (231 and Olga Kenzior unpublished data). The possible functions of polyomavirus Tags acetylation are discussed.

The reason why BKV fails to productively infect murine cells remains unclear. Through collaboration with Drs. Heinz-Peter Nasheuer and Michael Imperiale, combining the use of *in vitro* and *in vivo* DNA replication systems and ex vivo infection model, the restriction of BKV DNA replication in murine system has been shown to involve incompatibity of BKV Tag with murine pol-α primase and interaction of inhibitory cellular noncoding RNAs with BKV NCCR (135, 214, 215), summarized in Chapter 4. This also indicates that in addition to transcription factors/co-factors, cellular noncoding RNAs also regulate BKV DNA replication through NCCR.

To relate all the above observations to BKV reactivation in PVAN, we developed the hypothesis that ischemia/reperfusion injury reactivates BKV DNA replication in kidney allografts(78, 102). Chapter 5 demonstrates our observation that hypoxia can stimulate BKV DNA replication in cultured human kidney tubular epithelial cells. Accordingly, a model for BKV reactivation in PVAN is proposed to tie up all observations related with BKV NCCR: ischemia/reperfusion injury of kidney allografts changes the activity/expression of transcription factors (NFIs, HATs, etc.) and/or small noncoding RNAs in kidney tubular epithelial cells, which stimulates BKV replication and promotes BKV reactivation in PVAN. Other possibilities and the limitation of current studies are also discussed; and future directions are proposed.

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CHAPTER 2

Stimulation of BKV DNA replication by NFI-family transcription factors

INTRODUCTION

Human polyomavirus BK (BKV) persistently infects 80~90% people during early childhood without causing overt disease(41, 73, 74, 128). BKV DNA has been detected in multiple organs including liver, lung, brain, tonsil, etc., but the kidney is the primary site for its persistent infection (31, 62). BKV reactivation in kidney allografts of renal transplantation patients causes polyomavirus associated nephropathy (PVAN), a major source of allograft loss. During the asymptomatic persistent infection, BKV is maintained in normal kidneys, perhaps as episomes(31, 62), at < 0.01 copy/cell, and increases to >1000 copies/cell in allograft kidneys of PVAN patients (113). How BKV establishes its persistent infection and reactivates in allograft kidney remains undefined.

As with SV40 and mouse polyomavirus (mPyV), BKV DNA replication and gene transcription are controlled by the viral noncoding control region (NCCR). Within the NCCR, the core origin is the minimal sequence for DNA replication, flanked by an early origin flanking sequence (EF) and a late core origin flanking sequence ("enhancer"), to which transcription factors bind (90, 92) (Figure 2.1A). Remarkably, in late stage PVAN patients (53) or during passage of virus in cell culture(59, 123, 133), the BKV enhancer rearranges by duplication, deletion and insertion of four

sequence blocks, termed P_{68}, Q_{39}, R_{63}, S_{63} from archetype BKV. The archetype BKV (e.g. WW, Dik) with single copy linear configuration of P_{68}-Q_{39}-R_{63}- S_{63} is the form that persists among most people (Figure 2.1C). The relation of enhancer rearrangements to PVAN pathogenesis is unclear. However, it has been shown that rearranged strains replicate more efficiently than archetype BKV in cell culture, probably because the change of *cis*-elements in the enhancer provides an advantage for viral replication (19, 53, 108). This is also observed with JC virus (JCV), another closely related human polyomavirus that causes progressive multifocal encephalopathy (PML) in immunosuppressed and AIDS patients(34, 52), suggesting the *cis*-elements in the enhancer are important for viral reactivation and replication.

The interaction of cellular *trans*- acting elements with viral *cis*-acting elements responsible for replication is important for BKV persistent infection and reactivation. Studies of mPyV indicated that specific *cis*-elements in the mPyV enhancer regulate acute and persistent infection *in vivo* (117-119); and the enhancer is required for mPyV replication *in vivo* and stimulates SV40 DNA replication *in vivo* and *in vitro*(37, 57, 58). We have previously shown that substitution of the BKV enhancer with the mPyV enhancer significantly changes the efficiency of BKV DNA replication in cultured human kidney tubular epithelial cells (82); and the *cis*-elements in the BKV NCCR is partially responsible for the restriction of BKV DNA replication in murine cells (135). These observations support the suggestion that the enhancer is important for replication *in vivo*, but more detailed study is needed to understand the function of individual elements in the BKV NCCR and how they interact with host cellular factors to facilitate establishment of persistent infections and reactivation.

Binding sites for cellular transcription factors NFI(22-24, 39, 83), Sp1(22, 39, 83), NFAT(70), AP1(23, 39, 83), Smad3(1), ERE and GRE/PRE(91), p53(130), NFkB(51) and C/EBP(51) have been identified in the BKV enhancers and evidence provided for their importance. Putative binding sites for Ets-1, PEA3, AP-2, CREB, GM-CSF also have been predicted (90, 114), but need for their function is not clear. Notably, multiple NFI binding sites have been identified in the

BKV archetype (and rearranged) enhancers (22, 39, 83). Each block of P,Q,R,S contains at least one NFI site, thus all strains of BKV have multiple NFI sites. Particularly, the two NFI sites in P block (P_{24-37}) and P-Q junction (P_{68}-Q_{13}) (Figure 2.1C) are present in almost all strains (19, 53, 92, 108, 109, 114), suggesting these NFI sites might be essential for BKV replication. NFI sites have been shown to up-regulate or down-regulate BKV early/late promoter activity depending on the enhancer configuration and cell types (22-24, 45, 54, 76), however, a function of NFI sites in archetype BKV NCCR for DNA replication has not been defined, in part because the archetype BKV does not grow in cell culture(122).

NFI is a family of transcription factors highly conserved in all vertebrates that bind to "$TGGN_{5\sim7}$ GCCAA" sequences in many cellular and viral promoters (35, 55, 56). NFI has four isotypes, termed NFIA, NFIB, NFIC and NFIX (or NFID), encoded by four different genes (35, 55). These NFI isotypes have a highly homologous N-terminal DNA binding/dimerization domain while their C-terminal transactivation domains are completely different(55). The NFI isotypes can form homo- and hetero-dimers and carry out distinct functions(27, 55). Expression of NFI is ubiquitous, but expression pattern of NFI isotypes is distinct in different types of cells and changes during cell differentiation and embryonic development (26, 77, 111, 120). The function of NFI on gene transcription can be either stimulatory or inhibitory, and the mechanisms involved are complex, including: to activate transcription by direct interaction with basal transcription factors (TFIIB, TBP)(72, 138), by displacing repressive histones (3, 47, 116)and interacting with other transcription factors(32, 36) or HAT (histone acetyltransferase)(28, 79); and repressing transcription by competition with other transcription factors(12, 105, 106, 139) or other unknown mechanisms.

NFI was originally identified in HeLa nuclear extracts as a cellular factor that stimulates adenovirus DNA replication by recruitment of adenovirus DNA polymerase to the origin of replication(18, 29, 35, 99, 104). Recent studies indicate that it also cooperates with Oct-1 transcription factors to facilitate adenovirus initiation of replication by bending origin DNA (102).

Multiple NFI sites were identified on JCV enhancers and expression of different NFI isotypes determines the tropism of JCV(94, 115, 129). In addition, NFI sites were also identified in transcription control regions of many other viruses, including MMTV(11, 136, 137), HPV(7, 8, 107), HSV-1(78), CMV(6, 25, 48, 61) and the LTRs of MLV (43, 44, 50, 124), as being important for regulation of viral gene expression.

Here, we provide evidence for the function of NFI sites in BKV replication: NF1 sites in *cis*-stimulate BKV DNA replication, and mutation of NFI sites in the BKV enhancer diminish BKV DNA replication in competitive DNA replication assays. NFI interacts with Tag and the p58 subunit of DNA polymerase-α primase (pol- α primase), as detected by co-immunoprecipitation (Co-IP) assays. NFIC/CTF-1 stimulates BKV DNA replication *in vitro* when pol-a primase is limiting. We propose that NFI stimulates BKV DNA replication by recruitment of Tag and/or pol-α primase to replication origin, and may stimulate their activities.

MATERIALS AND METHODS

Plasmids. Test templates pUC-wt-BKV and pUC-Δen-BKV were generated from insertion of PCR fragments of intact NCCR (positions 5031 to 282) and NCCR without enhancer (positions 5031 to 32) of archetype BKV (Dik strain kindly provided by J. Lednicky; GenBank Accession #AB211369) into the XmaI/PstI sites of pUC18. pUC-6mtNFIs-BKV was generated by ligation of synthesized mutant BKV NCCR(GenScript) into XmaI/PstI of pUC18. pUC-5mtNFIsW1-BKV, pUC-5mtNFIsW2-BKV, pUC-5mtNFIsW3-BKV and pUC-5mtNFIsW6-BKV were mutated from pUC-6mtNFIs-BKV with relevant oligos using QuickChange Site-Directed mutagenesis kit (Stratagene). To construct test templates pUC-BKV-1fNFI, pUC-BKV-1rNFI, pUC-BKV-2rNFI, pUC-BKV-4rNFI, synthetic oligos "CACATGGAATGTAGCCAAAACTGCA" and "GTTTTGGCTACATTCCATGTGTGCA" were annealed and self-ligated before inserted into PstI site of pUC-Δen-BKV; and clones were screened by sequencing. Competition templates pBC-wt-BKV, pBC-BKV-en and pBC-BKV-ori were generated by insertion of PCR fragments of whole

NCCR (positions 5031 to 282), enhancer (position 33-282) and core origin (positions 5103 to 32) of BKV (Dik) into XmaI/PstI, NotI/PstI and NotI/XhoI sites of pBC-Sk(+) respectively. pBC-BKV-A89G, pBC-BKV-A143G, pBC-BKV-A141T were mutated from pBC-wt-BKV with relevant oligos using QuickChange Site-Directed mutagenesis kit (Stratagene).

The mammalian expression vector for BKV Tag is pCMV-BKT-Flag containing no SV40 origin as described (82). pCH-mNFIA1.1, pCH-mNFIB2, pCH-mNFIC2, pCH-mNFIX2 and pCH-empty were kindly provided by Richard Gronostajski (27). pCH-hNFIC/CTF1 was generated by insertion of the human NFIC/CTF-1 cDNA amplified by PCR from pCMV-CTF-1ΔUTR provided by Nicolas Mermod (87) using primers "5-AGCTGGGCCCATGGATGAGTTCCAC-3" and "5-TTGCGCTAGCCTATCCCAGATACCAGGAC-3" into NheI/ApaI sites of pCH-empty vector. pUbi-HA-Gal4dbd was provided by Olga Kenzior.

Bacteria expression vectors for truncated BKV Tag are pGEX3X-BKTHD, pGEX3X-BKTHDHR, pGEX3X-BKTHR. They were generated by insertion of PCR amplified fragments into EcoRI/BamHI sites of pGEX3X vector. Primers used for PCR are: BKTHD "ATAGGATCCCAGGCTTAAAGGAGCATGATTTTAAC" and "CGGCCAATTCTTAATCAAGAATACATTTCCCCATG"; BKTHDHR ""ATAGGATCCCAGGCTTAAAGGAGCATGATTTTAAC" and " ACGCGAATTCTTATTTTGGGGGTGGTGTTTTAG"; BKTHR "ATATGGATCCCAATTACAAGAGAAGAGGATTCAG" and " ACGCGAATTCTTATTTTGGGGGTGGTGTTTTAG".

Cell cultures and antibodies

HK-2 cells (human proximal tubular epithelial cells) were cultured in RPMI medium 1640 (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone), 4 mM L-glutamine and 100U/ml penicillin and 100g/ml streptomycin (Lonza). HEK293 cells (human embryonic kidney

epithelial cells) and HeLa cells were cultured Dulbecco's modified Eagle's medium (DMEM; low glucose) (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone), 4 mM L-glutamine and 100U/ml penicillin and 100g/ml streptomycin (Lonza). RPTECs (renal primary tubular epithelial cells) were culture in Renal Epithelial Growth Medium with supplements provided (Lonza). All cells were grown at 37°C with 5% CO2 in a humidified incubator.

***In vivo* DNA replication assays**

HK-2 cells were seeded in 12-wells plates ($1X10^5$ cells/well), and incubated overnight at 37°C. Cells were transiently tranfected with 1.0ug total DNA and 3ul FugeneHD transfection reagent (Roche) in 1000ul RPMI medium 1640 supplemented with 10% fetal bovine serum (Hyclone), 4 mM L-glutamine without antibiotics. Similarly, human HEK 293 cells were seeded in 12-well plates ($4X\ 10^5$ cells/well), and incubated overnight at 37°C. Cells were transfected with 0.7ug total DNA with 2ul LipofectAMINE and 5ul PLUS reagent (Invitrogen). After incubating cells with a DNA: LipofectAMINE and PLUS mixture for 4 to 5 h in 500ul of serum-free DMEM, the transfection solution was replaced with 1 ml of DMEM containing 10% FBS. The exact amount of templates and Tag expression vectors are indicated in each experiment's description; and total of amount of DNA were kept constant (1.0ug for HK-2 cells and 0.7ug for HEK293 cells) by adding pBC-SK(+) or pUC18 empty vectors. Cells were harvested at 48 h P.T (post-transfection), and low-molecular-weight (LMW) DNAs were extracted following the Hirt protocol(66) and purified with Promega Miniprep columns. The purified LMW DNAs were digested with EcoRI to linearize the plasmid, and digested with DpnI to distinguish input from replicated DNA in Tango Buffer (Fermentas). The DpnI- resistant DNA was resolved from digested DNA by agarose gel electrophoresis (1%). After capillary transfer of the DNA to a nylon membrane, DpnI-resistant DNA was detected by Southern blotting with a biotinylated probe of the BKV core origin (~80 nucleotides) and visualized by chemiluminescent nucleic acid detection (Pierce).

***In vitro* monopolymerase assays**

The *in vitro* monopolymerase assays were performed by Dr. Irina Tikhanovich as described (82, 135). Each reaction was supplemented with 0.5 mg of pUC-wt-BKV template DNA, 50 ng topoisomerase I, 1 mg RPA, 100 ng or ?ng pol-α primase and in 30 mM HEPES pH 7.8, 7 mM MgAc, 0.1 mM EGTA, 0.5 mM DTT, 200 uM each UTP, GTP, and CTP, 4 mM ATP, 100 uM each dATP, dGTP, and dTTP, and 10 uM dCTP, 40 mM creatine phosphate, 1 ug creatine kinase, 0.1 mg/ml heat-treated BSA, and 5 uCi [α^{32}P]-dCTP (3000 Ci/mmol, Perkin-Elmer) in 40 ml. The NFIC/CTF-1(Abcam) was added to the assay as indicated in the figure legends. Purified BKV Tag (0.2 mg) was added to start the reaction as indicated, and after incubation for 60 min at 37°C, the reaction products were precipitated with cold 10% (w/v) TCA containing 2.5% (w/v) sodium pyrophosphate and spotted on glass fiber filters (GF/C; Whatman), washed with 1 M HCl and analyzed by scintillation counting.

Competitive EMSA assays

Competitive EMSA assays were performed following published procedures (20) with a few modifications: 4pmol of competitor oligos, 2ug of HeLa nuclear extracts (sc-2120, Santa Cruz) or 0.25ug of purified hNFIC/CTF-1 (Abcam), 50ng of poly(dI.dC) were incubated in 1X HEPES Binding Buffer (25mM HEPES, pH7.5, 6mM $MgCl_2$, 0.5mM EDTA, 0.5 mM DTT, 50mM NaCl, 0.5mM phenylmethylsulfonyl fluoride (PMSF), 5% Glycerol) for 10 minutes at room-temperature (R.T); then, 20fmol of Biotin-labeled probe of NFI site was added into the reaction and incubated for 20 minutes at R.T. For antibody super-shift assays, 1ul of NFI antibody (sc-5567, Santa Cruz) was added after the binding reaction and incubated for another 10 minutes at R.T. The binding reaction product was fractionated in 5% non-denaturing polyacrylamide gel (pre-run in 0.5XTBE at 100V for 30minute) in 0.5XTBE at 100V until the bromophenol blue in the loading buffer reached bottom of gel. The fractionated probe in the gel was transferred to nylon member in 0.5XTBE at 65V for 30min and UV cross-linked for 15 minute and visualized by chemiluminescent nucleic acid detection (Pierce).

Preparation of nuclear extracts

To prepare large amount of HEK293 cell nuclear extracts for pull-down assays, 10 liters of HEK293 growing in Joklik's modified MEM medium supplemented with 5% new born calf serum to the density of $0.88X10^6$ cells/ml were purchased from The National Cell Culture Center(NCCC) and the nuclear extracts were prepared with the following steps: the cells pellet was washed in 5 packed cell volumn (pcv) of ice-cold hypotonic buffer (10mM HEPES, pH7.9, 1.5mM $MgCl_2$, 10mMKCl, 0.2mM PMSF, 0.5mM dithiothreitol (DTT)) and centrifuged with Beckman GH-3.7 rotor at 3000rpm for 5minutes in 4°C; pelleted cells were re-suspended in 3 pcv of ice-cold hypotonic buffer and allowed to swell on ice for 10 minutes ; swollen cells were transferred to a pre-chilled glass Dounce tissue grinder (Wheaton Scientific) and homogenized with 10-15 strokes using type B pestle and centrifuged with Beckman GH-3.7 rotor at 3500rpm for 15 minutes in 4°C; supernatant were removed and the pellets were re-suspended in 1 packed nuclear volume (pnv) of low salt buffer (20mM HEPES, pH7.9, 25% glycerol, 1.5mM $MgCl_2$, 0.2mM EDTA, 0.2mM PMSF, 0.5mM DTT); 0.183 pnv of 5M NaCl was added dropwise into the re-suspended nuclei while mixing gently by swirling; the nuclear extraction mixture was mixed gently on a rotating platform for 30 minutes in 4°C and centrifuged at 16000rpm(37,000g) in Sorvall SA-600 rotor for 1 hour in 4°C. The supernatants were collected and dialyzed in dialysis buffer (20mM HEPES, pH7.9, 20% glycerol, 5mM NaCl, 0.2mM EDTA, 0.2mM PMSF, 0.5mM DTT). The final concentration of extracts was 1.4ug/ul determined by Bradford protein assay.

Small-scale preparations of HK-2, HeLa, RPTEC and HEK293 cells nuclear extracts for Western Blotting were made using Nuclear Extract Kits (Active Motif) following the procedures recommended in the instruction manual.

***In vitro* pull-down assays**

Full-length Tag pull-down assays: The full-length BKV Tag with Flag epitope tag was expressed using Bac-to-Bac Baculovirus System (Invitrogen). $1.5X10^7$ of Hi-Five insect cells were seeded in two 150mm flasks and cultured overnight in 27°C. One flask of Hi-Five cells were infected at MOI=10 with baculovirus encoding Tag-Flag grown in Sf9 cells; the other was not infected. Both infected cells and non-infected Hi-Five cells were harvested 48 hours post-infection (P.I) and lysed in 1ml of 0.5% NP-40 Lysis Buffer (50mM Tris-Cl, pH7.5, 150mM NaCl, 5mM KCl, 1.0mM $MgCl_2$, 0.5% NP-40, 10% glycerol, 1X PhosSTOP phosphatase inhibitors (Roche), 1X Complete protease inhibitor cocktail (Roche)) by incubation on a rotating platform in 4°C for 30 minutes; then the lysate was transferred to a glass Dounce tissue grinder (Wheaton Scientific) and homogenized with 20 strokes using the type A pestle; the lysate was cleared by centrifugation at 12000rpm(20,000g) in Sorvall SA-600 rotor for 30 minutes in 4°C; the supernatant was incubated with 60ul of Anti-Flag M2 Affinity Gel (Sigma) by rotating at 4°C for 2 hours; after incubation, the Anti-Flag M2 Affinity Gel was washed three times with 1ml of ice-cold PBS. Then, each of the gels incubated with extracts of infected and uninfected Hi-Five cells was incubated with 1ml of HEK293 cells nuclear extracts (1.4ug/ul) plus 1X Complete protease inhibitor cocktail (Roche) by rotating in 4°C for 12 hours. The gel was washed three times with 1ml of ice-cold PBS and boiled 5 minutes with 50ul of 1X SDS sample buffer (63mM Tris-Cl, pH6.8, 10% Glycerol, 2% SDS, 50 mM DTT, 0.0025% Bromophenol Blue).

Truncated Tags pull-down assays: Various truncated GST-BKTag were expressed in Rossetta™ 2 *E.coli* cells (Novagen), a BL21 strain carrying tRNAs for rare codons to enhance expression of eukaryotic genes in bacteria. Transformed Rossetta™ 2 cells were cultured in LB medium supplemented with 34ug/ml chloramphenicol and 100ug/ml ampicillin in a shaking platform at 225rpm in 25°C. Expression of the fusion proteins was induced with 1mM isopropyl-1-thio-D-galactopyranoside (IPTG) when the density of *E.coli* cells reached 0.4-0.5 (OD_{600}). After induction, *E.coli* cells were cultured in 25°C with 225rpm shaking for 20 hours. To purify the proteins, *E. coli* cells were lysed by sonication (twice, 300 seconds each, 20% duty cycle, maximum power) in Buffer L1 (50 mM Tris-Cl, pH 8.0, 250 mM NaCl, and 1 mM dithiothreitol

(DTT), 10% glycerol, 1mM PMSF, Complete protease inhibitor cocktail (Roche)). 0.1% NP-40 was added to the lysates after sonication and incubated on ice for 10 minutes. After separating the soluble supernatant of the cell lysates from the pellet by centrifugation at 12000rpm(20,000g) in Sorvall SA-600 rotor for 30 minutes in 4°C, the fusion proteins in the supernatant fraction were incubated with glutathione affinity beads (Amersham Biosciences) in 4°C for 2 hours followed by washing the beads twice with 20 bed volume of buffer L1. A small portion (1/20) of the beads for each fusion protein were boiled with SDS sample buffer to check the quantity of bind fusion proteins by SDS-PAGE followed by Commassie Staining. Beads with approximately equal amount of each fusion protein (~50ug) were incubated with 800ul of HEK293 nuclear extract (1.4ug/ul) supplemented with Complete protease inhibitor cocktail (Roche) by rotating in 4°C for 12 hours. The beads were washed 5 times with 1ml of buffer L1, and then boiled 5 minutes with 100ul 1X SDS sample buffer.

All samples were fractionated by SDS-PAGE and analyzed by Commassie Blue Staining and Western Blotting, followed by Western Bloting with antibodies obtained from Santa Cruz or Cell Signalling.

Co-immunoprecipitation assays

HEK293 cells (approximately $6X10^6$) were transfected with expression vectors (as indicated in particular experiments) by using LipofectAMINE and PLUS reagent (Invitrogen) in a 100-mm-diameter plate following procedures similar to those described in " *in vivo* DNA replication assays". 48 hours P.T, the cells were lysed with 750ul of 1% Triton Lysis Buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 30uM ethidium bromide, 1 mM phenylmethylsulfonyl fluoride (PMSF), Complete protease inhibitor cocktail (Roche), 1X PhosSTOP phosphatase inhibitors (Roche)) by rotating for 1 h at 4°C, and lysates were cleared by centrifugation at 12000rpm(20,000g) in a Sorvall SA-600 rotor for 30 minutes at 4°C. A sample (10ul) was taken for protein input control, and the remaining supernatants were incubated with 50ul of Anti-Flag M2

Affinity Gel (Sigma) or 50ul of Anti-HA Affinity Matrix (Roche) by rotating at 4°C for 2 hours, followed by four washes with 1ml of 1% Triton Lysis Buffer. Following the last wash, pelleted beads were suspended in 40ul of 1X SDS sample buffer, boiled for 5 minutes. Samples of 20ul of the supernatant were loaded for SDS-PAGE and Western Blotting analysis using specific antibodies.

Protein stability assays

HKE293 cells were seeded in 24-wells plate ($2X10^5$ cells/well) and incubated overnight at 37°C. Cells were transfected with 0.15ug of Tag expression vectors (pCMV-BKT-Flag) and NFI expression vectors (pCH- empty, NFIA1.1, NFIB2, NFIC2 and NFIX2) by using LipofectAMINE and PLUS reagent (Invitrogen) following similar procedures described in "*in vivo* DNA replication assays".24 hours post transfection (P.T.), equal volume of fresh complete DMEM containing 200ug/ml cycloheximide (CHX) was added into the culture. Cells were harvested at 0 hour, 12 hours, 24 hours, 36 hours after CHX-treatment and frozen in liquid N_2 and stored in -80°C. All cells were lysed with 100ul of 1% Triton Lysis Buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), Complete protease inhibitor cocktail (Roche), 1X PhosSTOP phosphatase inhibitors (Roche)) for 1 h at 4°C, and lysates were cleared by centrifugation at 12000rpm(20,000g) in Sorvall SA-600 rotor for 30 minutes in 4°C. Supernatants were boiled with equal volume of 2X SDS sample buffer for 5 minutes. 20ul of the boiled samples were loaded for SDS-PAGE and Western Blotting analysis using anti-Flag, anti-HA and anti-β actin antibodies. Density of each blot was quantified using Quantity One® 1-D Analysis Software (Bio-Rad).

RESULTS

Enhancer in *cis*- stimulates BKV DNA replication when template and Tag are limiting.

The viral enhancer is required for mPyV DNA replication *in vivo*(110) and stimulates SV40 DNA replication *in vivo* and *in vitro* (37, 57, 58) and BKV viruses with rearranged enhancers have higher replication efficiency than those with the archetype enhancer (19, 53, 108). To test if the enhancer also stimulates BKV DNA replication, replication of BKV templates with the enhancer (archetype Dik strain) (134) (pUC-wt-BKV) and templates without enhancer (pUC-Δen-BKV) was compared in transiently transfected HK-2 cells (Figure 2.1A). BKV Tag was expressed with expression vector pCMV-BKT-Flag. The enhancer *in cis*- strongly stimulated BKV template replication when low amounts (10ng) of Tag expression vector and template (10ng) were used (Figure 2.1B). The stimulatory effect of the enhancer was diminished with higher amounts of input template (>100ng) and Tag expression vector (>50ng) (data not shown). Similar observations were also obtained from HEK293 cells and PRTEC cells (data not shown). These indicate that the enhancer in *cis*- stimulates BKV DNA replication when the amounts of template and Tag are low. Transcription factors binding to enhancer might be responsible for the stimulatory effect of enhancer (Figure 2.1C). We seek to determine which transcription factor(s) play such a role.

Figure 2.1 Stimulation of BKV DNA replication by enhancer. (A). Construction of replication templates. pUC-wt-BKV is template with whole BKV NCCR (Dik strain); pUC-Δen-BKV is template without enhancer. EF is early flanking sequence; Core-Ori is core origin of replication. (B). Southern Blot results of replication of BKV test templates with and without enhancer. Arrow indicates the position of replicated templates; solid line indicates the DpnI digested input template. (C). Sequence of BKV archetype enhancer and transcription factor binding sites.

Figure 2.1A

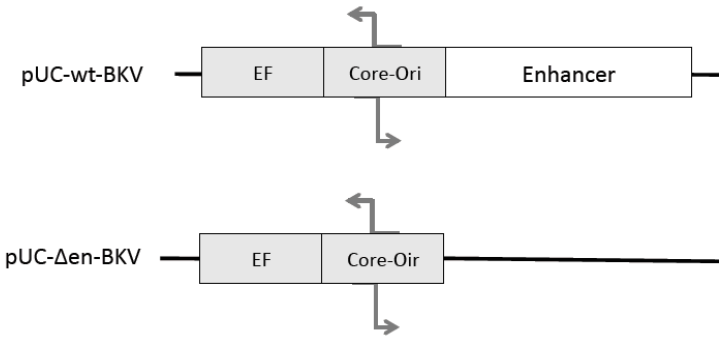


Figure 2.1B

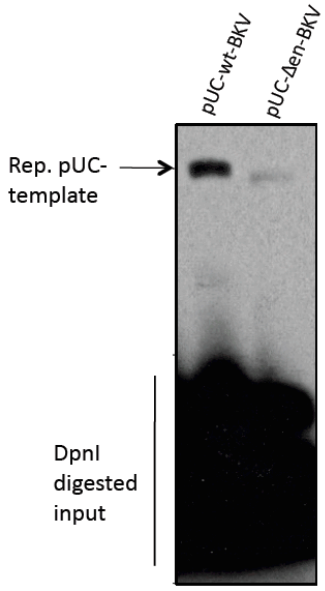
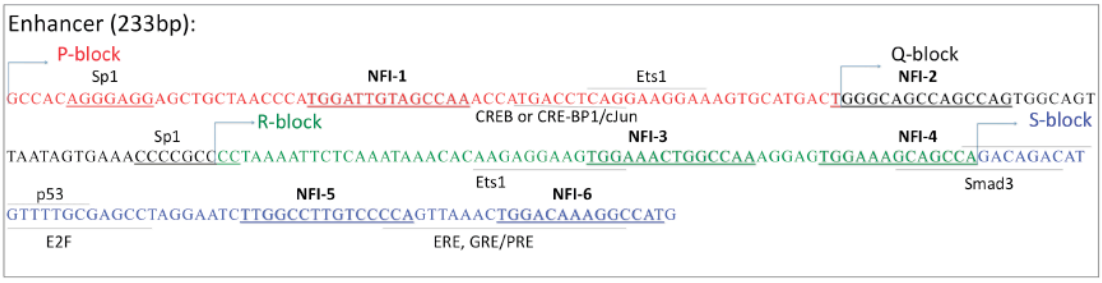


Figure 2.1C



NFI and BKV Tag form complex *in vitro.*

Transcription factor c-Jun has been shown to stimulate mPyV DNA replication through recruitment of Tag to origin of replication(68). Transcription factors in HEK293 nuclear extracts able to interact with BKV Tag were pulled-down by full-length Flag-tagged BKV Tag (BKT-Flag) and identified by Western Blotting with specific antibodies: NFI, p53, Sp1, c-Jun, but not Ets-1, CREB, NFkB p65, Smad3, CBP/p300 and PCAF/GCN5 formed stable complexes with BKV Tag (Figure 2.2A). Which domains of BKV Tag interact with these transcription factors was tested by GST pull-down assays with GST-tagged truncated BKV Tags (Figure 2.2B). The Tag Helicase/ATPase domain was able to strongly pull-down NFI, p53, c-jun, but not CREB, NFkB or PCAF, and very weakly, if at all, Sp1 (Figure 2.2C lane 4 and 5). These observations are consistent with previous reports that SV40 Tag interacts with p53(71, 81, 125), Sp1(69) and c-Jun(13); and also consistent with reports that BKV Tag, NFI and Sp1 complex with p53(130) and p53 interacts with the helicase domain of Tag (71, 81, 125) and Sp1 interacts with origin binding domain (OBD) of Tag(69). None of the transcription factors tested here interact with the Tag C-terminal region (Figure 2.2C lane 3). Truncated Tags did not pull down any of the factors that were not in complex with full-length Tag (Figure 2.2A, C; data not shown).

NFI was pulled-down by both full-length and the helicase domain of BKV Tag. Specific antibodies for NFIA, NFIB, NFIC were used to distinguish the isotype- specific interaction of NFI with BKV Tag. Only NFIA was detected in the input of HEK293 nuclear extracts and the pulled-down fractions with Tag helicase domain (Figure 2.2B panel NFIA). NFIB and NFIC were not detectable in HEK293 nuclear extracts; and NFIX was not tested due to poor quality of antibody (data not shown). Thus, the isotype NFIA forms a stable complex with BKV Tag *in vitro* through Tag's helicase/ATPase domain; whether other NFI isotypes stably interact with Tag needs to be tested with additional assays.

Figure 2.2 In vitro pull-down assays (A) Flag pull-down assays to identify transcription factors interacting with BKV Tag. Lane1, input of HEK293 cell nuclear extracts; lane 2, BKV full-length Tag pulled-down fraction; lane 3, mock pull-down fraction in the absence of Tag. Number of specific transcription factors binding sites on BKV archetype enhancer is illustrated by number of "+"; "+/-" stands for a half conserved site; NA stands for not available.

Figure 2.2A

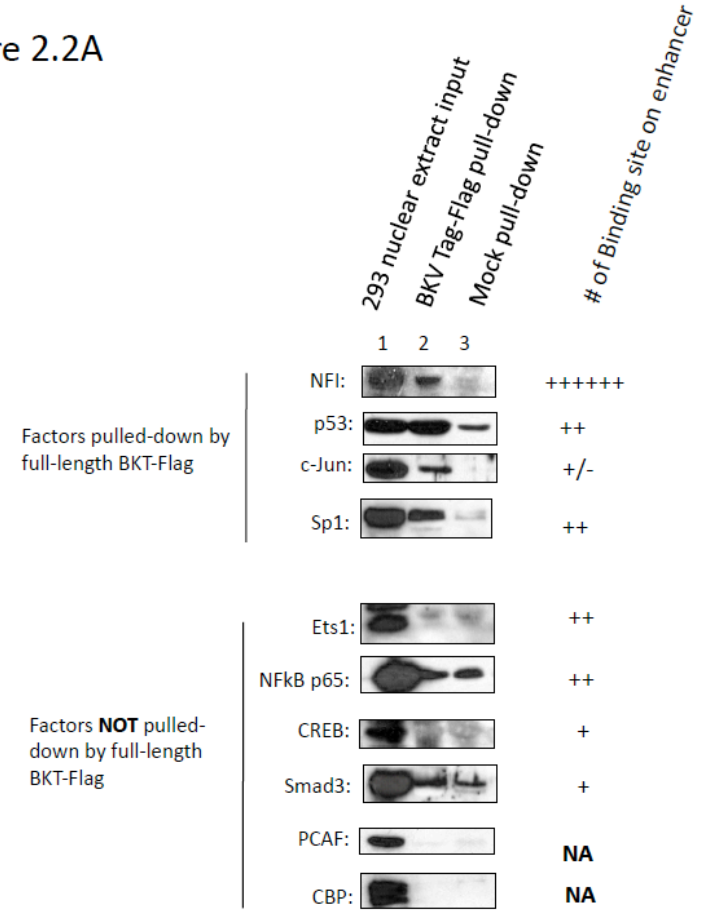

293 nuclear extract input
BKV Tag-Flag pull-down
Mock pull-down
# of Binding site on enhancer
1 2 3
Factors pulled-down by full-length BKT-Flag
NFI:
++++++
p53:
++
c-Jun:
+/-
Sp1:
++
Factors NOT pulled-down by full-length BKT-Flag
Ets1:
++
NFkB p65:
++
CREB:
+
Smad3:
+
PCAF:
NA
CBP:
NA

Figure 2.2 (B). Illustration of truncation mutants of BKV Tag using in GST pull-down assays. (C). GST pull-down assays to determine interaction of specific Tag truncation mutants with transcription factors. Lane 1, HEK293 nuclear extract input; lane 2, GST pull-down fraction control; lane 3, GST tagged BKT C-terminal region (Cter) pull-down fraction; lane 4, GST tagged BKT helicase domain (HD) pull-down fraction; lane 5, GST tagged BKV Tag helicase and host range domain (HDHR) pull-down fraction.

Figure 2.2B

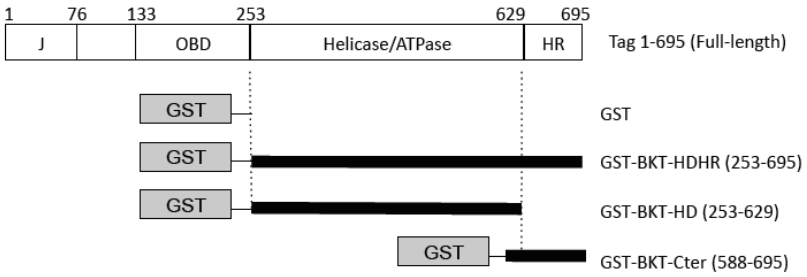
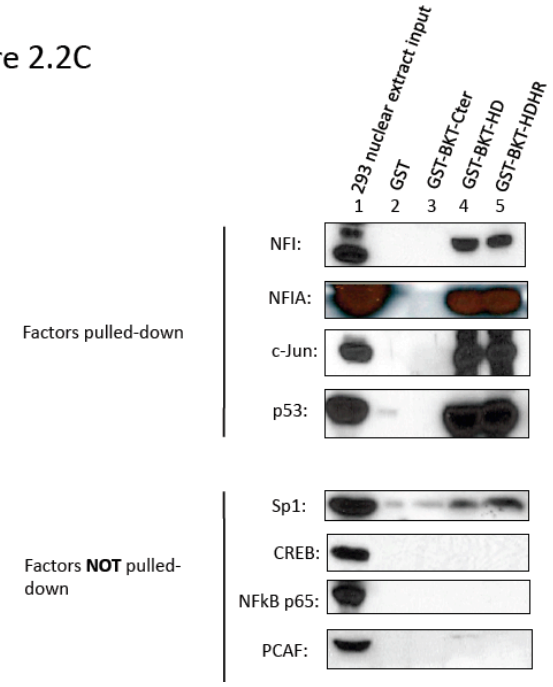


Figure 2.2C



NFI interacts with BKV Tag *in vivo*.

To further confirm that NFI interacts with BKV Tag, we attempted to detect their interaction by Co-Immunoprecipitation(Co-IP) assays. Since NFI expression is ubiquitous, we first compared the expression of NFIA, NFIB and NFIC in four types of cells including HEK293, HK-2 (human proximal tubular epithelial cells), HeLa and PRTEC (primary renal tubular epithelial cells), which are used in our laboratory to study BKV replication. We found that expression patterns of NFI isotypes differ between these cells (Figure 2.3A-D). A summary of the expression in the four types of cells is provided in Table 1. All three isotypes are highly expressed in HeLa cells (lane 4 of Figure 2.3B-D), but are poorly expressed in PRTEC cells (lane1 of Figure 2.3B-D). In HEK293 cells, only NFIA is well expressed, and the expression of NFIB and NFIC is barely detectable (lane 3 of Figure 2.3B-D). In HK-2 cells, NFIA and NFIB are well expressed, but NFIC expression is low (lane2 of Figure 2.3B-D). Since the expression pattern of NFI isotypes seems to be the simplest in HEK293 cells and their transfection efficiency is high, we used HEK293 cells to study *in vivo* interaction between BKV Tag and NFI by Co-IP assays following ectopic expression of HA-tagged NFI isotypes and Flag-tagged BKV Tag in HEK293 cells.

When NFI isotypes were precipitated by anti-HA (Figure 2.4A), BKT-Flag was co-precipitated effectively with HA-NFIB (Figure 2.4A lane 3), while the co-precipitation of BKT-Flag with other HA-NFI isotypes was much weaker (Figure 2.4A lane 2, 4, 5). In the reciprocal strategy to precipitate Tag by anti-Flag, more NFIB was co-precipitated by Tag than other NFI isotypes (Figure 2.4B, lane 2-5). No co-precipitation was detected when BKT-Flag was co-expressed with an empty NFI expression vector (pCH-empty) (Figure 2.4A lane1; Figure 2.4B lane1) or another vector expressing an unrelated HA-tagged Gal4 DNA binding domain (pUbi-HA-Gal4dbd), even though the expression of Tag was much higher (Figure 2.4A lane6; Figure 2.4B lane6). Endogenous NFkB p65 was included as an internal control to check the quality of *in vivo* assays and to see if the *in vivo* results are in agreement with *in vitro* results: NFkB p65 co-precipitated very weakly, if at all, with NFIC and NFIX, and not with NFIA and NFIB (Figure 2.4A, panel “WB:

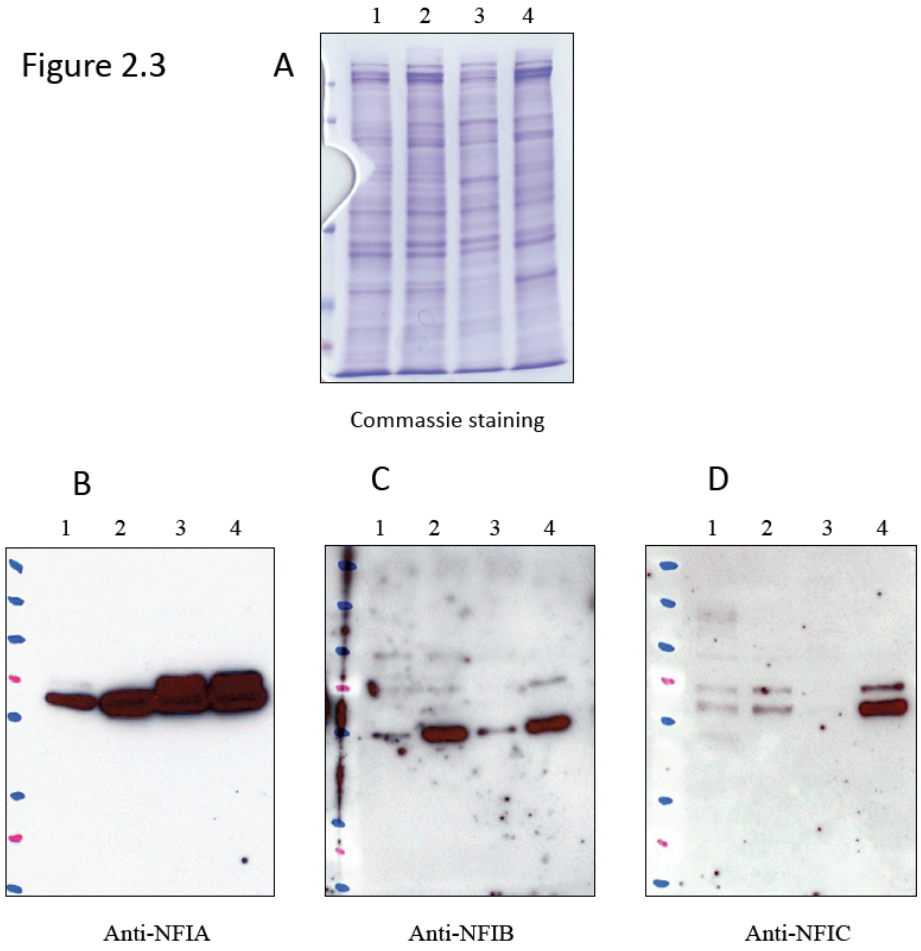
anti-NFkB p65"), which is a totally different pattern compared with BKT-Flag (Figure 2.4A, panel "WB: anti-Flag"), indicating they are in different protein complexes. But NFkB p65 did not co-precipitate with BKT-Flag (Figure 2.4B, panel "WB: anti-NFkB p65"), which is consistent with the *in vitro* results. Similar results have been obtained from at least two independent repeats.

It is notable that the expression of BKT-Flag from the CMV promoter (pCMV-BKT-Flag) was differentially affected by different NFI isotypes (Figure 2.4A, "Input" panel for "WB: Anti-Flag", lane 1-5), which was confirmed with CMV-luciferase reporter assay (Figure 2.4C). However, the expression of BKT-Flag does not seems to be regulated by NFI only at the transcription level, because the CMV promoter activity determined in reporter assays (Figure 2.4C) cannot explain the huge difference at the protein level in Western Blot (Figure 2.4A, "Input" panel for "WB: Anti-Flag", lane 1-5). The interaction of Tag with different NFI isotypes might influence the stability of BKT-Flag. Cyclohexmide (CHX) chase assays indicated that the stability of Tag is greatly reduced by co-expression of NFIA and NFIX, while co-expression of NFIB and NFIC have little effect on Tag's protein stability (Figure 2.4D).

Figure 2.3 Expression of NFI isotypes in different cells. Lane 1, 2, 3 and 4 are nuclear extracts of PRTEC, HK-2, HEK293 and HeLa cells respectively. (A). commassie staining of nuclear extracts from four types of cells; (B). expression of NFIA; (C). expression NFIB; (D). expression of NFIC.

Table 1. Summery of NFI isotype expression in four types of cells.

Figure 2.3



Differential expression of endogenous NFI isotypes in different cells

Table 1

Cell types	NFIA	NFIB	NFIC	NFIX
1. PRTEC	++	+	+	N.A
2. HK-2	++++	+++++	+	N.A
3. HEK293	+++++	+	-	N.A
4. Hela	+++++	+++++	+++++	N.A

Figure 2.4 Co-Immunoprecipitation (Co-IP) assays to detect Tag interaction with NFI transcription factors. BKV Tag expression vector is co-transfected with expression vectors for NFI isotypes (pCH-A, B, C,X or empty) or control vector expressing Gal4 DNA binding domain (pUbi-HA-Gal4dbd). (A). Immunopreciptiation of HA-tagged NFIs and detection of co-precipitation of Flag-tagged Tag; (B). Immunoprecipitation of Flag-tagged Tag and detection of co-precipitation of HA-tagged NFI.

Figure 2.4A

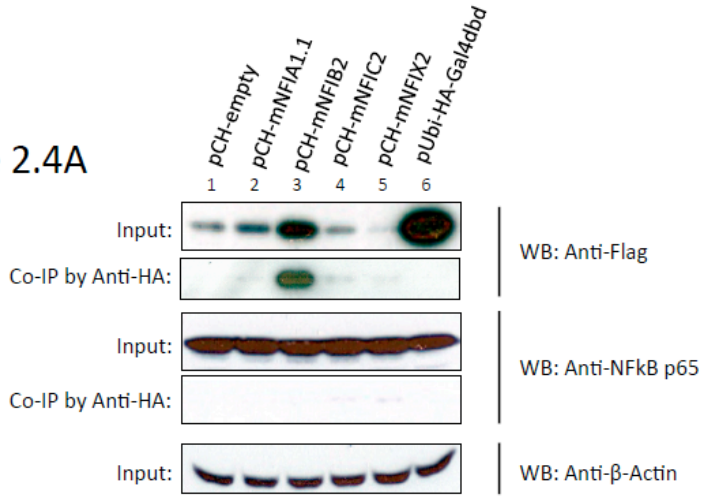


Figure 2.4B

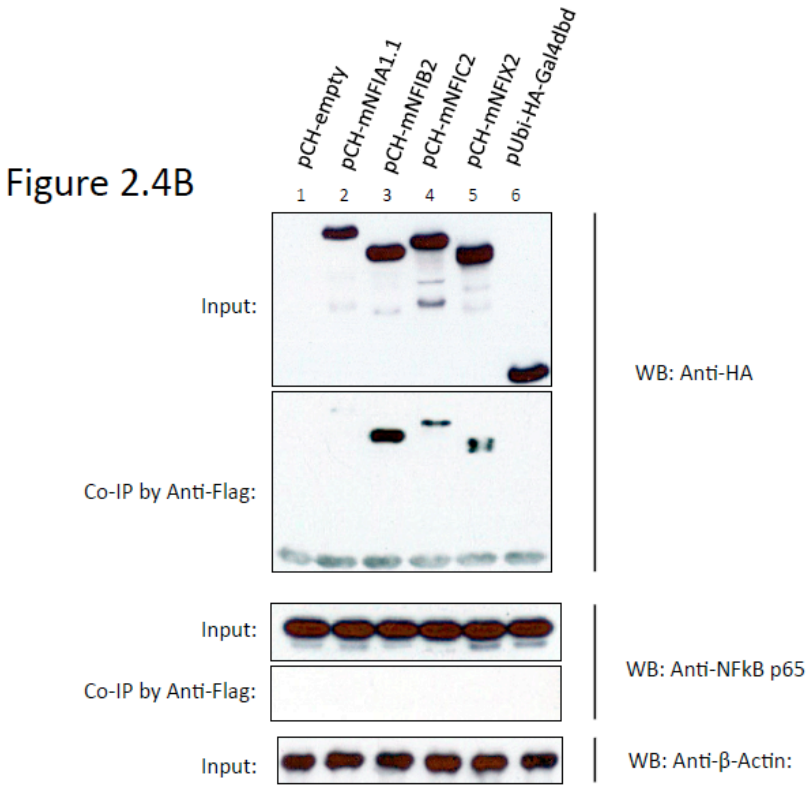


Figure 2.4 (C). CMV-luciferase assays: HKE293 cells were transfected with pCH-NFIs and pCMV-Ren at same ratio as pCH-NFIs and pCMV-BKT-Flag in Co-IP assay. Expression of luciferase was measured 24 hours P.T; (D). Cyclohexmide (CHX) chase assays. Transfected cells were treated with 200ug/ml CHX 24 hour P.T. Expression of Flag-tagged Tag, HA-tagged NFIs and β-actin were detected with Western Blotting; (E). Quantification of CHX chase assay results. Bend density of Flag-Tag was normalized with β-actin; the normalized density at 0 hour CHX treatment was arbitrarily set as “1” and the normalized densities of later time points were calculated relatively to the normalized density of 0 hour.

Figure 2.4C

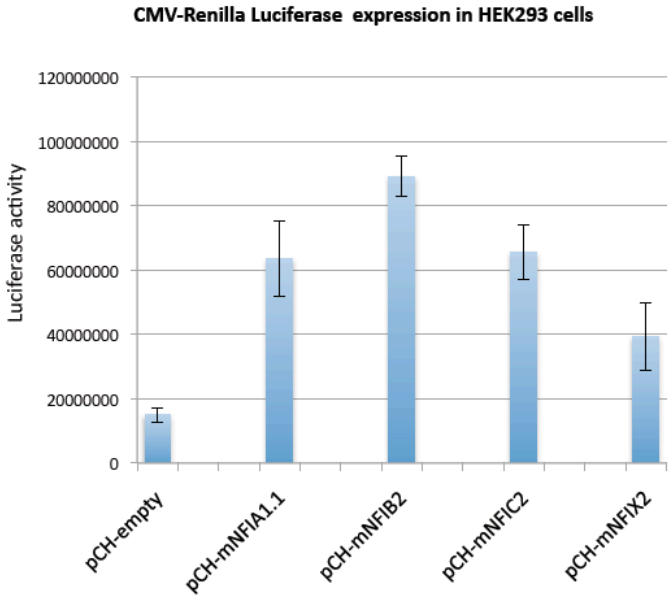


Figure 2.4D

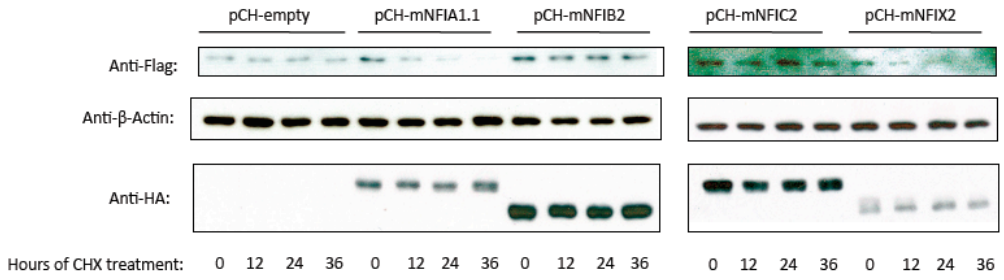
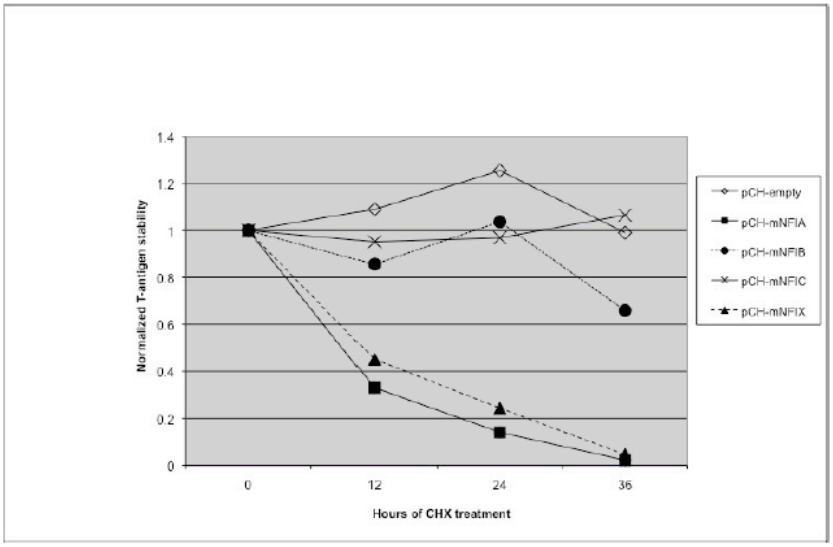


Figure 2.4E



Synthetic NFI sites in *cis*- stimulate BKV DNA replication

To determine if NFI stimulates BKV Tag dependent DNA replication in the absence of interference from other *cis*-elements in BKV enhancer, the enhancer was substituted by synthetic NFI binding sites in different orientations and copy numbers (Figure 2.5A). The first NFI site in the P block of archetype BKV enhancer (NFI-1) was used as the synthetic NFI binding site. This NFI site was confirmed with EMSA (electrophoretic mobility shift assay) assays as being able to bind NF1 (data not shown). The synthetic NFI sites stimulate BKV DNA replication (Figure 2.5B lane 2-5); and stimulation of BKV template replication by NFI sites correlates with copy number (Figure 2.5B lane 6-8). Orientation of NFI sites seems not to be important (Figure 2.5B lane 3, 4). Although NFI sites stimulate BKV DNA replication, the replication of templates with multiple NFI sites was weaker than replication of BKV templates with the wildtype enhancer, suggesting other factors in addition to NFI contribute to the stimulatory effect.

Figure 2.5 DNA replication of BKV templates with synthetic NFI sites. (A). Illustration of design of BKV templates with synthetic NFI sites. (B). Southern Blot of DNA replication assays with BKV templates with synthetic NFI sites.

Figure 2.5A

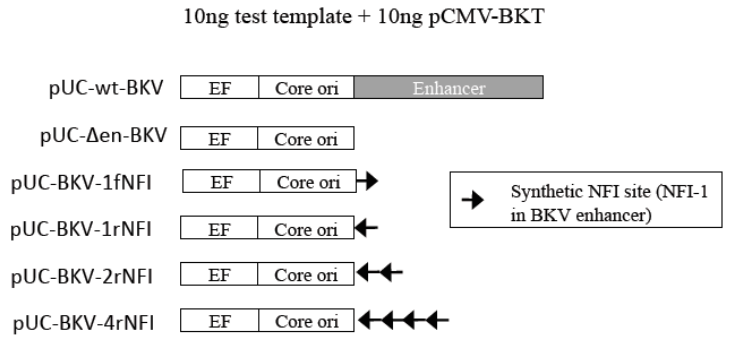
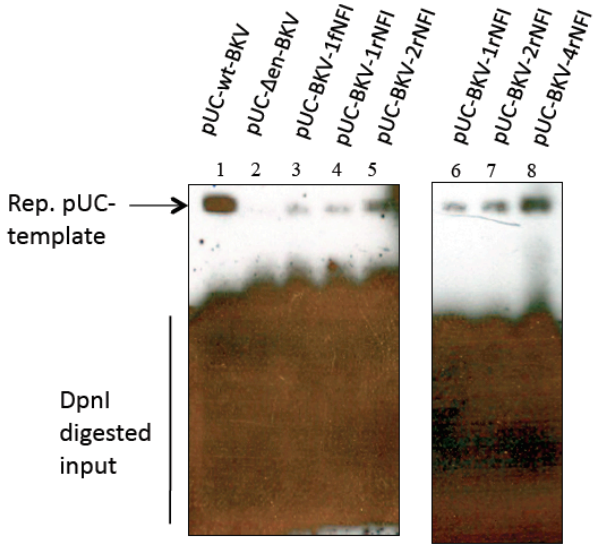


Figure 2.5B



BKV enhancer with mutant NFI sites is defective in NFI binding

Six NFI binding sites have been identified in the BKV archetype enhancer (Figure 2.1C). To evaluate NFI binding to these sites, a competitive EMSA assay was developed. The Biotin-labeled oligo conaining the NFI site of the adenovirus ITR (inverted terminal repeat) with high binding affinity (80) was incubated with HeLa nuclear extracts. A shifted band of oligo caused by NFI binding was observed in the absence of competitor (Figure 2.6B, lane 1, 6). Adding excess unlabeled oligo with the same adenovirus ITR NFI site fully competed the binding by NFI in HeLa extracts (Figure 2.6B, lane 2, 7). However, the competitor with a point mutation in adenovirus ITR failed to compete for NFI binding (Figure 2.6B, lane3). Adding anti-NFI antibody to the binding reaction caused "super-shifting" (dash-arrow in Figure 2.6B, lane4), indicating the band shifting was caused by NFI binding, but not by nonspecific protein binding. An excess of BKV enhancer PCR fragment also competed for NFI binding (Figure 2.6B, lane5), confirming that NFI can bind to BKV enhancer (Dik). Oligos of six NFI binding sites in BKV enhancer (Figure 2.6A) were all able to compete for NFI binding, but with different efficiencies (Figure 2.6B, lane 8-13), suggesting these NFI sites have different affinities for NFI. Sites closer to the core origin seem to have higher affinities than distal sites.

Point mutations were introduced into both arms of the NFI consensus binding sequence "TGGN$_{5\sim7}$ GCCAA" of each of the six sites to ensure complete disruption of NFI binding to the BKV enhancer (Dik) (Figure 2.6A) (86, 121, 126). The design of the mutations was confirmed with MatInspector (112) to ensure that no significant new transcription factor binding sites were created by introducing mutations in the BKV enhancer. NFI binding to all mutant NFI sites are defective in competitive EMSA assays (Figure 2.6C). The competitive binding of mutant BKV enhancer competitor with mutations on all six NFI sites (6mtNFIs BK enhancer) was greatly diminished compared with the wild type enhancer competitor (Figure 2.6D, lane 2,3), but some weak competitive effect was still observed with excessive molar amount of mutant BKV enhancer,

which became more obvious when the amount of mutant BKV enhancer competitor was increased (Figure 2.6D, lane 3-5). However, the mutant enhancer competitor was almost completely defective in binding when purified hNFIC/CTF1 was used instead of HeLa nuclear extracts (Figure 2.6E). These results suggest that mutation of six NFI sites greatly reduces NFI binding to BKV enhancer, however, some cryptic NFI binding site(s) may still be present in the mutant BKV enhancer, to which NFI binds cooperatively with other transcription factors in the nuclear extracts.

Figure 2.6 EMSA assays to detect NFI binding to BKV NCCR. (A). Sequences of oligos for NFI sites used in EMSA assays. NFI binding motifs were underlined. Point mutations were indicated above the mutated positions; (B). Competitive EMSA assays using the NFI site in Adenovirus inverted terminal repeat (Ad ITR) as probe. Lane 1, in the absence of competitor; lane 2, oligo of NFI site Ad ITR as competitor; lane 3, oligo for mutant NFI site Ad ITR as competitor; lane 4, in the absence of a competitor, but incubated with anti-NFI antibody for 10min after binding reaction; lane 5, PCR fragments of BKV enhancer as competitor; lane 6, same as lane 1; lane 7, same as lane 2; lane 8-13, oligos of NFI sites in BKV enhancer as competitors. Positions of free probe and shifted probe are indicated with arrows.

Figure 2.6A

```
                          C             A
NFI-1: TAACCCATGGAATGTAGCCAAACCATGA

                          C              G  T
NFI-2: GCATGACTGGGCAGCCAGCCAGTGGCAGT

                          C             G  T
NFI-3: GAGGAAGTGGAAACTGGCCAAAGGAGTG

                          C             A
NFI-4: AAAGGAGTGGAAAGCAGCCAGACAGACA

                          C              G
NFI-5: AGGAATCTTGGCCTTGTCCCCAGTTAAAC

                                       G  T
NFI-6: GTTAAACTGGACAAAGGCCATGGTTCTG
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Figure 2.6B

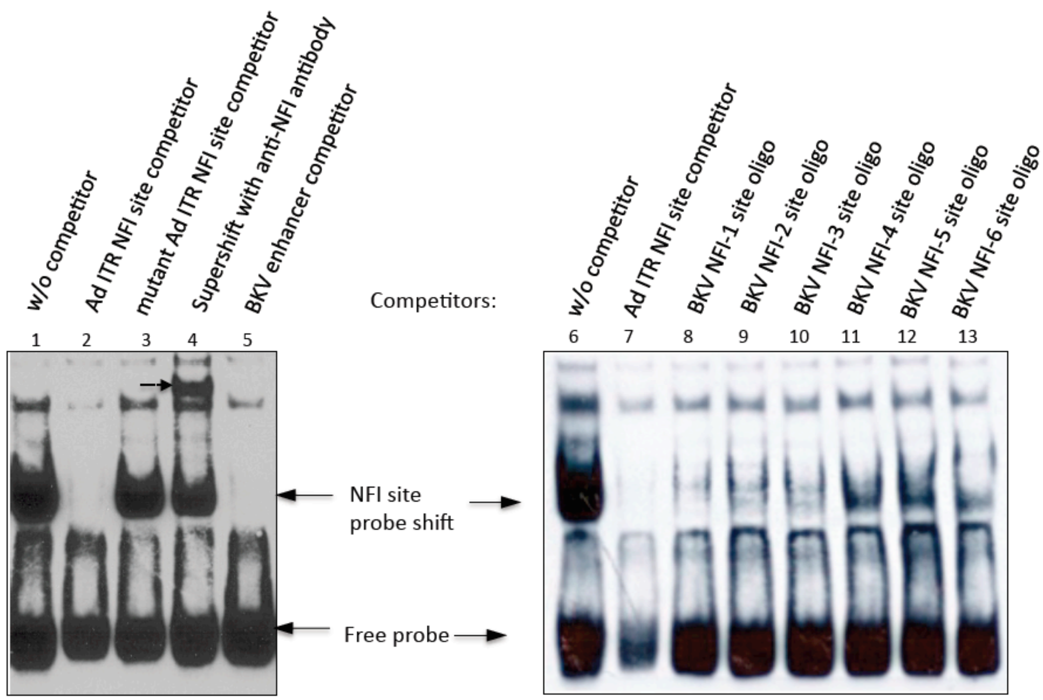


Figure 2.6 (C). Competitive EMSA assays with wildtype (wt) NFI sites and mutant (mt) NFI sites in BKV enhancer. (D). Competitive EMSA assays with mutant BKV enhancer containing six mutated NFI sites (6mtNFIs). Lane 1, in the absence of competitor; lane 2, with wildtype BKV enhancer PCR fragment as competitor; lane 3-5, with increasing amount of 6mtNFIs mutant BKV enhancer PCR fragment as competitor. (E). Competitive EMSA assays using purified NFIC/CTF1 instead of HeLa nuclear extracts. Lane 1, in the absence of competitor; lane 2, wildtype BKV enhancer PCR fragment as competitor; lane 3, 6mtNFIs mutant BKV enhancer PCR fragment as competitor.

Figure 2.6C

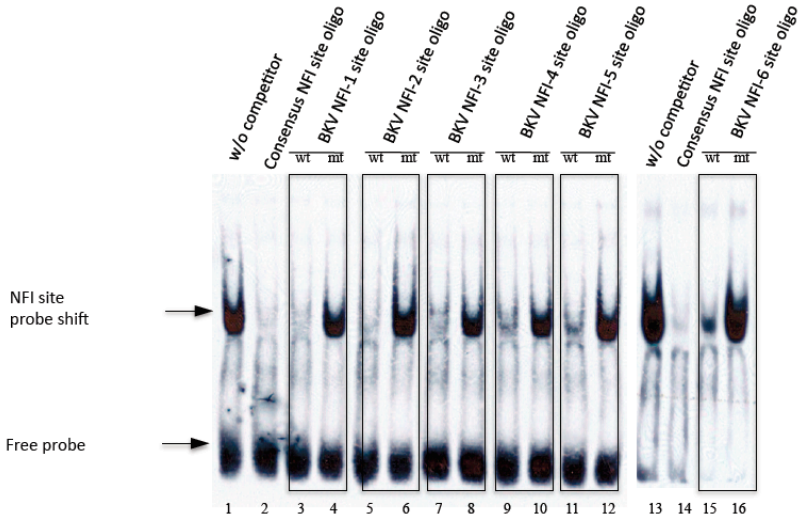


Figure 2.6D

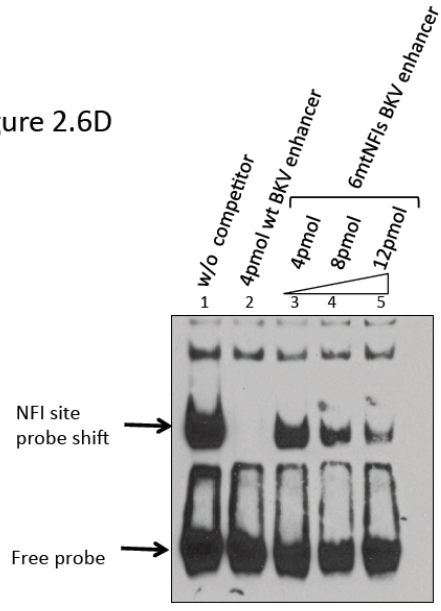
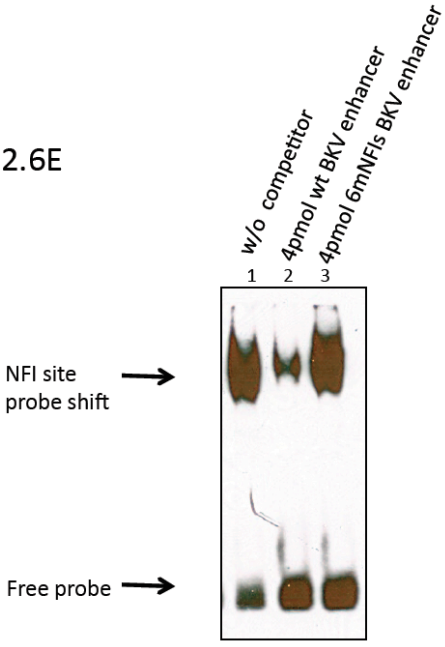


Figure 2.6E



NFI sites on BKV enhancer stimulate BKV DNA replication *in vivo*

Initially, replication of the mutant BKV template with mutations in all six NFI sites (pUC-6mtNFIs-BKV) was compared with replication of wildtype template (pUC-wt-BKV) in HK-2 cells. However, the mutant template replicated with similar efficiency as the wildtype template (data not shown). We speculate this might be due to the NFI binding to cryptic binding sites, or to redundant stimulatory effects from other transcription factors.

An alternative competitive DNA replication assay was used to test the effect of mutations in the NFI sites. A replication competitor (pBC-wt-BKV) with wildtype BKV NCCR in a pBC-backbone was introduced and transfected together with the test template (in a pUC-backbone) and a Tag expression vector (pCMV-BKT-Flag). In the presence of the competitor (pBC-wt-BKV), replication of mutant template (pUC-6mtNFIs-BKV) was greatly reduced compared to replication of the wildtype template (Figure 2.7A, lane 1, 3), and similar to the replication of template without an enhancer (Figure 2.7A, lane 2). However, another template with mutations in putative Ets-1 binding sites (pUC-mtEts1s-BKV) replicated at same efficiency as wildtype template (Figure 2.7A, lane 1, 5), indicating Ets1 is not important for BKV DNA replication.

To determine which sites are mainly responsible for the stimulatory effect of NFI sites, several mutated NFI sites were changed to wildtype individually. Replication of mutant templates with only one wildtype NFI site suggests that NFI sites closer to core origin are more important for BKV DNA replication in the competitive assays (Figure 2.7B, lane 3-7).

The requirement for a competitor in the BKV DNA replication assay to observe the stimulatory effect of NFI sites suggests that NFI may help to recruit some limiting factors to the replication complex. Furthermore, that NFI sites closer to core origin are more important than distal sites suggests that NFI targets components of the initiation complex, including Tag, DNA polymerase-

α primase (pol-α primase), RPA and Topoisomerase I. We examine this possibility by the experiments described below.

Figure 2.7 Competitive DNA replication assays in the presence of a wildtype BKV template (pBC-wt-BKV) as competitor. Solid arrow indicates replicated test template; dashed arrow indicate replicated competitor template. (A). lane 1, wildtype BKV test template (pUC-wt-BKV); lane 2, enhancer deletion BKV test template (pUC-Δen-BKV); lane 3, mutant BKV template with six mutant NFI sites (pUC-6mtNFIs-BKV); lane 4, mutant BKV template with five mutant NFI sites (5mtNFIs) and the 6th NFI site is wildtype(W6) (pUC-5mtNFIsW6-BKV); lane 5, Ets1 sites mutant BKV template (pUC-mtEts1s-BKV). (B). lane 1, wildtype BKV test template (pUC-wt-BKV); lane 2, enhancer deletion BKV test template (pUC-Δen-BKV); lane 3, 6mtNFIs mutant BKV template (pUC-6mtNFIs-BKV); lane 4, 5mtNFIs mutant BKV template with the 1st NFI being wildtype(W1) (pUC-5mtNFIsW1-BKV); lane 5, 5mtNFIs mutant BKV template with the 2nd NFI being wildtype(W2) (pUC-5mtNFIsW2-BKV); lane 6, 5mtNFIs mutant BKV template with the 3rd NFI being wildtype(W3) (pUC-5mtNFIsW3-BKV); lane 7, 5mtNFIs mutant BKV template with the 6th NFI being wildtype(W6) (pUC-5mtNFIsW6-BKV).

Figure 2.7A

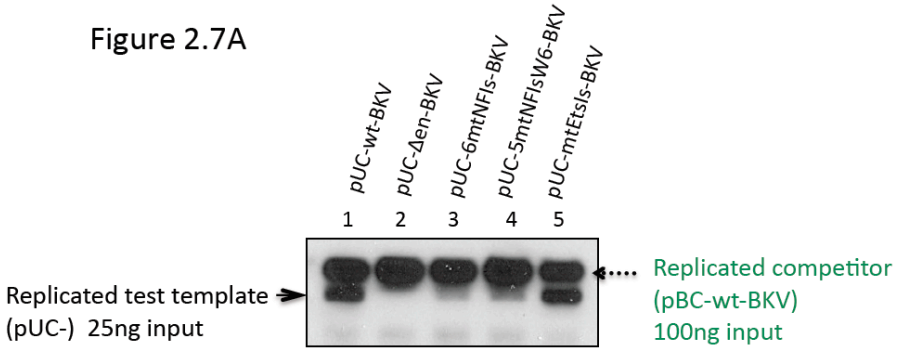
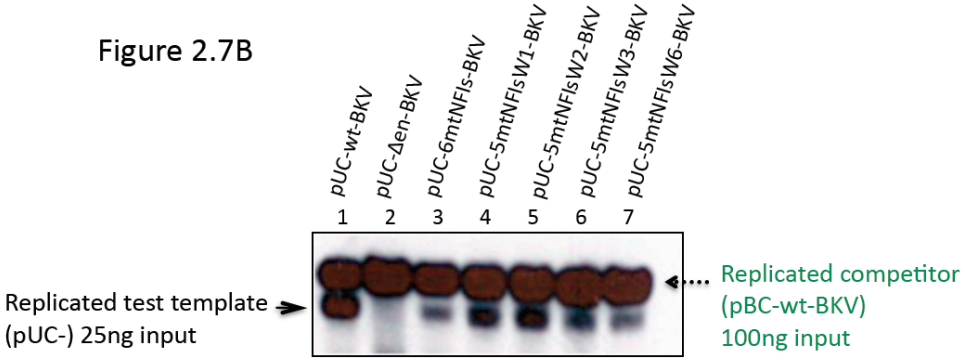


Figure 2.7B

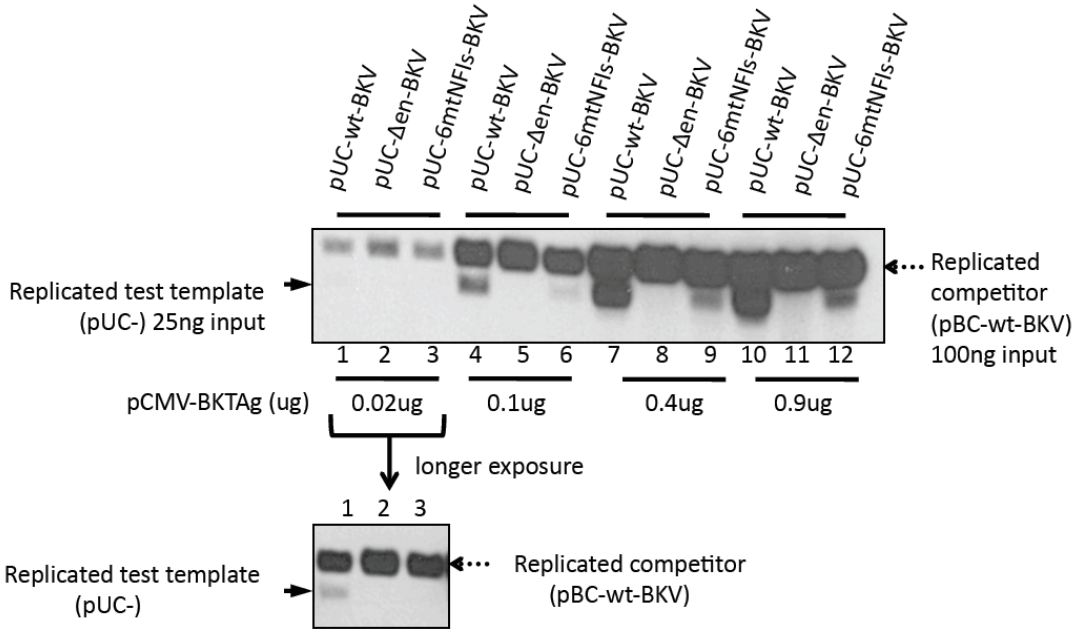


Tag is not the sole target for NFI stimulatory activity

That BKV Tag interacts with NFI suggests Tag might be the basis for the effect upon replication of mutating NF1 sites. To determine if BKV Tag is the limiting factor responsible for the observed stimulation of BKV DNA replication by NFI in competitive DNA replication assays (Figure 2.7A), BKV Tag was over-expressed in HK-2 cells and DNA replication of wildtype and NFI sites mutant templates was compared in the competitive replication assay. Surprisingly, the templates with mutant NFI sites (pUC-6mtNFIs-BKV) replicated much less efficiently than the wildtype template (pUC-wt-BKV) even when Tag was over-expressed (Figure 2.8, lane 7,9 and lane 10,12). The template with a deleted enhancer (pUC-Δen-BKV) also did not replicate when Tag was over-expressed (Figure 2.8, lane 8, 11), in contrast to its replication in the absence of a competitor (Figure 2.1). These observations suggest that Tag is not the sole limiting factor for NFI in competitive replication assays.

Figure 2.8. DNA replication assay in the presence of wildtype BKV competitor (pBC-wt-BKV) as a competitor while titrating the transient expression of BKTAg. The amount of transfected BKTAg expression vector is increased from 0.02ug to up to 0.9ug. Three test templates including wildtype BKV template (pUC-wt-BKV), enhancer deletion BKV template(pUC-Δen-BKV) and 6mtNFIs BKV template(pUC-6mtNFIs-BKV) are tested as indicated. Results of longer exposure for the first three lanes is included. Solid arrow indicates replicated test template; dashed arrow indicate replicated competitor template.

Figure 2.8



NFI stimulates BKV DNA replication by targeting component(s) of the host DNA replication machinery

To determine factor(s) targeted by NFI, several mutant competitors were constructed and assessed in replication assays: competitor containing only enhancer (pBC-BKV-en) did not replicate, due to the lack of core origin; competitor containing only core origin (pBC-BKV-ori) replicated very inefficiently (data not shown); three competitors containing the complete BKV NCCR, but each with a single point mutation in the AT-rich or EP region of core origin (pBC-BKV-A89G, pBC-BKV-A141T, pBC-BKV-A143G) replicated very inefficiently (Figure 2.9A). These mutant competitors were used in the competition DNA replication assay to examine how NFI sites in these mutant competitors affect templates replication. BKV Tag was over-expressed to exclude the possibility that Tag is limiting in the cells. Surprisingly, the enhancer (pBC-BKV-en), core origin (pBC-BKV-ori) and replication-defective competitors (pBK-BKV-A89G, pBK-BKV-A143G) are not effective competitors for the unknown cellular factor(s) that stimulates BKV DNA replication through NFI sites in competitive replication assays in the presence of the wildtype competitor (pBC-wt-BKV) (Figure 2.9B, lane 3-10). The difference in replication of wildtype and mutant templates was only observed in the presence of replication competent competitors (pBK-wt-BKV and pBK-BKV-A141T) (Figure 2.9B, lane 1-2 and 11-12). These observations suggest that the competitor in the replication assay must be able to replicate efficiently, suggesting the limiting factor might be a component(s) of the DNA replication machinery that acts beyond binding of Tag.

Figure 2.9 Competitive replication assays with mutant competitors. (A). Replication of mutant competitors with point mutations(A89G, A141T, A143G) in the core origin. (B). Competitive DNA replication assays to compare the replication of wildtype (wt) and NFI sites mutant template (6mtNFIs) in the presence of different competitors: lane 1-2, wildtype competitor (pBC-wt-BKV); lane 3-4, competitor containing only enhancer (pBC-BKV-en); lane 5-6, competitor containing only core origin (pBC-BKV-ori); lane 7-8, competitors with point mutation of A89G in core origin (pBC-BKV-A89G); lane 9-10, competitors with point mutation of A143G in core origin (pBC-BKV-A143G); lane 11-12, competitors with point mutation of A141T in core origin (pBC-BKV-A141T). Solid arrow indicates replicated test template; dashed arrow indicate replicated competitor template.

Figure 2.9A

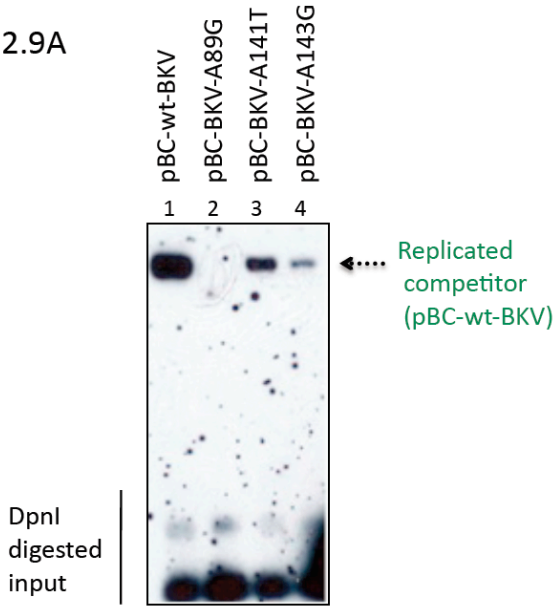
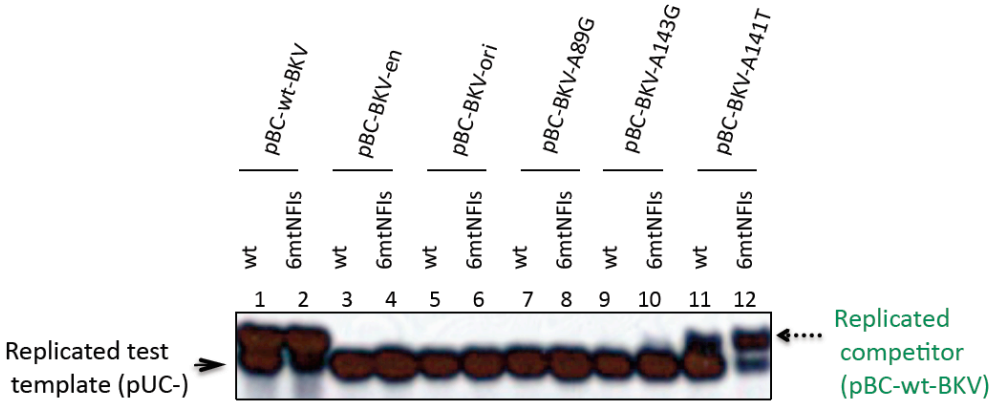


Figure 2.9B



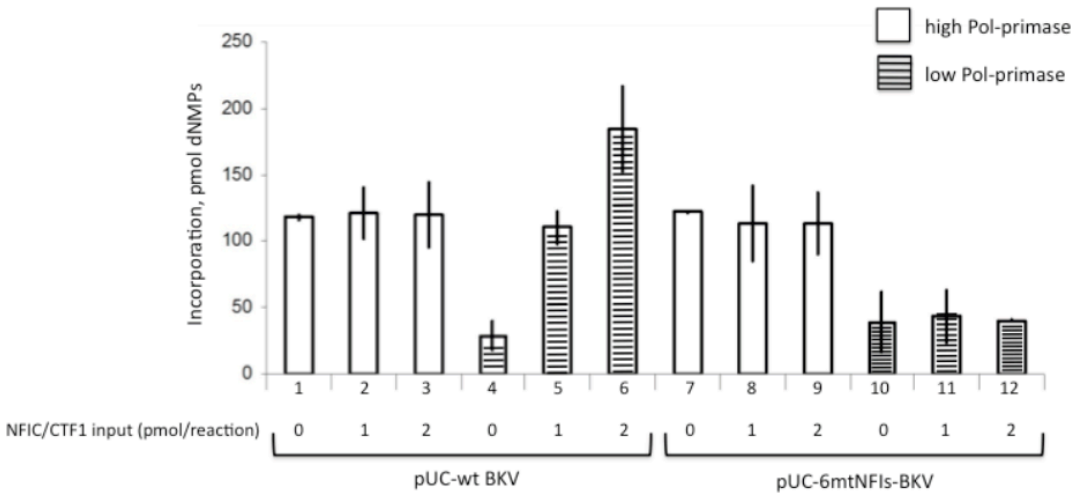
NFIC/CTF1 stimulates BKV DNA replication *in vitro* when DNA polymerase-α primase is limiting

NFI stimulate initiation of adenovirus DNA replication by recruiting adenovirus DNA polymerase to origin of replication (18, 29). The proline-rich transactivation domain of NFI (isotype NFIC/CTF1) stimulates SV40 DNA replication when tethered to the origin (100). This prompted us to assess whether NFIC/CTF1 can stimulate initiation of BKV DNA replication in the monopolymerase system that contains Pol-α primase, RPA and Topoisomerase I (82, 135) (Dr. Irina Tikhanovich did this experiment).

In support of NFI targeting a component of the monopolymerase assay, when pol-α primase was limiting, the initiation on wildtype BKV template was stimulated strongly by NFIC/CTF1 in a dose-dependent manner (Figure 2.10, lane 4-6). In contrast, with high levels of pol-α primase, adding NFIC/CTF1 had no effect in initiation on wildtype BKV template (Figure 2.10, lane 1-3). Furthermore, no stimulation was observed with the NFI binding mutant BKV template regardless of pol-α primase level (Figure 2.10, lane 7-12). This indicates that NFIC/CTF1 stimulates initiation of BKV DNA replication only when pol-α primase is limiting. And pol-α primase might be the limiting factor targeted by NFI to stimulate BKV DNA replication in the competitive replication assays.

Figure 2.10. Monopolymerase assays with purified NFIC/CTF1. Initiation of wildtype BKV template (pUC-wt-BKV) and NFI sites mutant BKV template (pUC-6mtNFIs-BKV) were tested with high (empty columns) and low (solid columns) amount of purified pol-α primase with increasing amount of purified NFIC/CTF1.

Figure 2.10

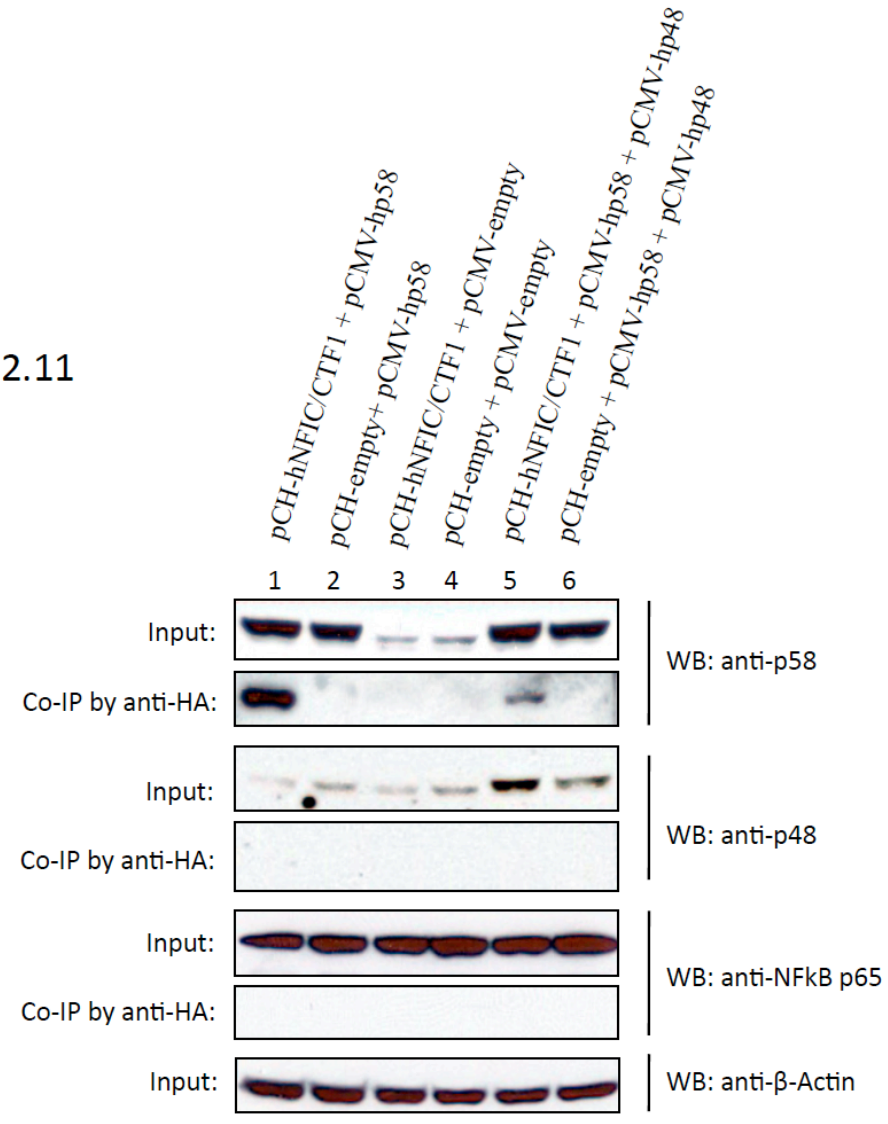


NFIC/CTF1 interacts with the p58 subunit of cellular DNA polymerase-α primase

DNA polymerases-α primase consists of four subunits: two smaller subunits, p48 and p58 constitute the primase; and two larger subunits, p68 and p180 constitute the DNA polymerase-α (88, 89). The p48 is the catalytic subunit of primase and p58 helps with nuclear translocation of p48 (89). HA tagged human NFIC/CTF was over-expressed with p58 alone or with p48 and p58 in HEK293 cells; the interaction of NFIC/CTF1 with p48 or p58 was examined with Co-IP assays. HA tagged human NFIC/CTF1 was immunoprecipitated with anti-HA. The p58 was co-precipitated with NFIC/CTF1 (Figure 2.11 lane 1, panel WB: anti-p58), but p58 co-precipitation was not detected in three control reactions, in which p58 (Figure 2.11 lane 2, panel WB: anti-p58) or NFIC/CTF1 (Figure 2.11, lane 3, panel WB: anti-p58) or neither (Figure 2.11, lane 4, panel WB: anti-p58) was expressed. Because p48 localizes in cytoplasm and its expression is very low without co-expression of p58 (data not shown), p48 and p58 were co-overexpressed in HEK293 cells with NFIC/CTF-1. However, no co-precipitation of p48 subunit could be detected (Figure 211 lane 5-6, panel WB: anti-p48), although a weak co-precipitation of p58 was observed (Figure 211 lane 5-6, panel WB: anti-p58), probably because most p58 subunit was in complex with over-expressed p48. This suggests that NFIC/CTF-1 and p48 might interact with the same domain of p58. No significant interaction between NFIC/CTF-1 and p180 or p68 was observed with Co-IP assays (data not shown).

Figure 2.11 Co-IP assays to detect interaction of NFI with primase. NFIC/CTF1 and p48/p58 subunits of pol-α primase were over-expressed in HEK293 cells as indicated: lane 1, NFIC/CTF1 and p58 subunit; lane 2, p58 subunit alone; lane 3, NFIC/CTF1 alone; lane 4, blank control; lane 5, NFIC/CTF1 and p48/p58; lane 6, p48/p58. Western Blotting with specific antibodies are indicated beside each panel. NFkB p65 was used as internal negative control; β-actin was used as loading control.

Figure 2.11



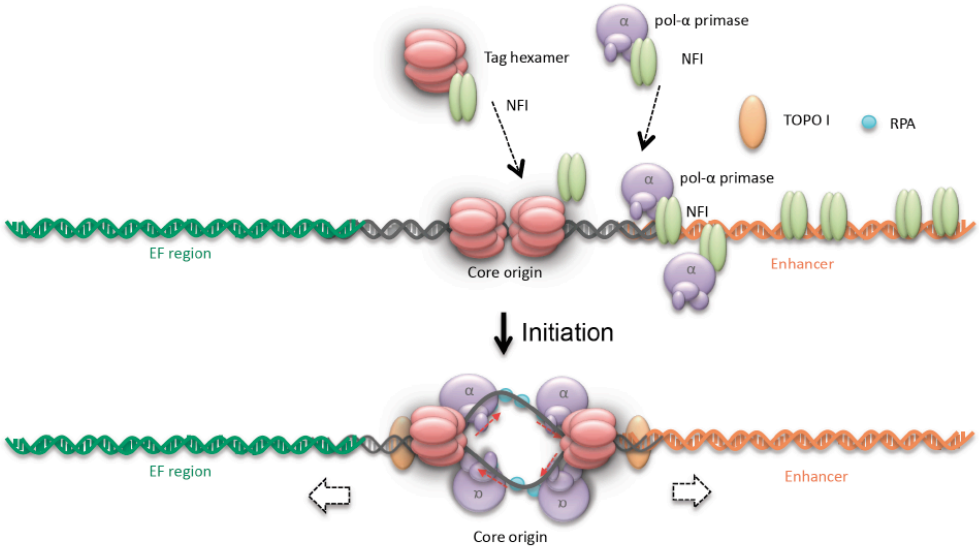
DISCUSSION

After almost two decades since the first report of PVAN, the mechanism for BKV reactivation still remains elusive. Immune suppression is partially responsible for reactivation of BKV (2, 15, 42, 60, 140). The involvement of cellular factors induced by the stress related injury, repair, regeneration and differentiation has been proposed (46, 63, 65). The viral NCCR is the docking site for cellular transcription factors. Understanding how *cis*-acting elements on NCCR regulates BKV replication might provide new insight to the mechanism of BKV reactivation and strategy to mitigate PVAN.

Here, we found transcription factor NFI binding sites in BKV NCCR in *cis*- stimulate BKV DNA replication *in vivo* and *in vitro* (Figure 2.5B, Figure 2.7A,B, Figure 2.10); NFI is in complex with BKV Tag (Figure 2.2A, B, Figure 2.4A, B) and pol-α primase (Figure 2.11 A,B). It is well established that NFI stimulates adenovirus (Ad2/5) DNA replication(80, 104) through recruitment of Ad pol-pTP complex (adenovirus DNA polymerase-preterminal protein) to replication origin (18, 29, 99) and/or stabilization of pre-initiation complex(98). According to these observations, we propose that NFI might also stimulate BKV DNA replication through recruitment of BKV Tag and pol-α primase to replication origin (Figure 2.12). In support of this, we found that NFI stimulated initiation of BKV DNA replication *in vitro* only at low concentration of pol-α primase, whereas at high concentration of pol-α primase no stimulation was observed (Figure 2.10, lane 1-6). This is reminiscent of the similar stimulation of adenovirus DNA replication *in vitro* by NFI, which was also dependent on concentration of pol-pTP (99). And this is also consistent with the results of *in vivo* replication showing that the stimulatory effect of NFI sites is observed only when a competitor is present (Figure 2.7A,B and data not shown). It seems that the competitor is competing for the factors that is/are component(s) of DNA replication machinery in addition to Tag (Figure 2.9B).

Figure 2.12 Proposed model for NFIs stimulating BKV DNA replication. When Tag and pol-α primase are limiting in the cells, NFIs help recruiting them to origin of replication through interaction and facilitate the assembly of initiation complex to promote DNA replication.

Figure 2.12



Six NFI sites in the BKV archetype NCCR were verified by EMSA assays (Figure 2.6A lane 8-13). We found these NFI sites not to be equally important for BKV DNA replication: sites closer to core origin have higher affinity to NFI (Figure 2.6A lane 8-13) and also have stronger stimulatory effect on BKV DNA replication than distal sites (Figure 2.7B, lane 4-7). These also support the hypothesis that NFI helps to recruit the components of DNA initiation complex to core origin. That NFI sites closer to core origin in P_{24-37} (NFI-1) and P_{68}-Q_{13} junction (NFI-2) have stronger stimulatory activity and almost all rearranged viruses contain the P block and P-Q junction region (53, 92, 108, 109, 114) suggest that these two NFI sites might be essential for efficient viral DNA replication *in vivo*. However, we do not exclude the possibility that these sites might have other functions in addition to stimulating viral DNA replication.

Other NFI sites in BKV enhancer (Figure 2.1C) might be *cis*-acting regulators for early/late gene transcription as reported previously (23, 24, 54, 76). It was determined that the 3rd NFI sites on archetype BKV enhancer (NFI-3) overlaps the initiation site for late transcription and represses the late promoter, providing a mechanism for early-late switch of viral gene expression (76). The NFI-4 site in R block overlaps a Smad3 site that activates the BKV early promoter in responds to TGF-β(1). Since NFI activity is also be regulated by TGF-β(4), NFI binding to NF-4 might regulate BKV replication in response to TGF- β induced in kidney allografts. The last two NFI sites (NFI-5 and NFI-6) at the late side of enhancer overlap an ERE (estrogen response element) and GRE/ERE (glucocorticoid/progesterone response element), both of which independently stimulate viral replication in transient transfection assays (91). Corticosteroids are used in kidney transplantation patients as anti-rejection treatment, which is a risk factor for development of PVAN in renal transplantation patients in addition to HLA-mismatch(64). And BKV reactivation has been reported extensively among pregnant women(14, 33, 127). So although NFI-5 and NFI-6 sites have no significant effect in our DNA replication assays, they might function as competitive inhibitors for hormone-activated nuclear receptors to prevent high-level replication during persistent infection.

The JC virus (JCV) archetype enhancer contains five NFI sites, all of which are highly conserved with NFI sites in archetype BKV, except that BKV has an additional NFI site (NFI-4) overlapping the Smad3 site(84). This suggests that NFI might be essential for replication and persistence of both BKV and JCV. The NFI site close to the core origin of JCV was shown to stimulate JCV DNA replication *in vivo* (132), perhaps through similar mechanism as proposed here for BKV. In support of this, analysis of JCV rearranged enhancers in PML patients also revealed a similar pattern as with rearranged BKV enhancers in PVAN patients that blocks close to core origin (A to C for JCV; P and P-Q junction for BKV), which contain the first two NFI sites, are usually preserved and duplicated (52, 53). NFIX expression is required for JCV propagation in permissive cells, while NFIA expression is inhibitory for JCV replication in non-permissive cell (93, 115, 129). Whether it is DNA replication or gene transcription of JCV that is regulated differently by distinct NFI isotypes is unknown. We have not been able to distinguish the function of different isotypes in the *in vivo* replication assays because endogenous NFI isotypes complicate the result interpretation and no BKV strains have been shown to display a specific tropism in cell culture. However, using the *in vitro* monopolymerase assay, we have defined the stimulatory activity of isotype NFIC/CTF-1 for initiation of BKV DNA replication. The role of other isotypes in DNA replication will be tested in similar systems in the future.

Although the NFI sites are not absolutely required for BKV DNA replication in DNA replication assays in the absence of a competitor (data not shown), they stimulate BKV DNA replication when Tag or pol-α primase is limiting (Figure 2.5B, Figure 2.7A). This stimulatory activity might be essential for reactivation, because latent BKV persists episomally in kidney tubular epithelial cells at less than one copy/cell and thus expresses limiting amount of Tag(31, 62, 113). And the tubular epithelial cells in normal kidney are terminally differentiated quiescent cells dividing at low rate (16, 103), most of which probably express low amounts of pol-α primase. Stress related signaling induced by kidney ischemeia/reperfusion injury or inflammatory response during kidney transplantation might change the NFI isotypes expression or their activity through post-translational modifications or interaction with other cellular factors, which may promote the NFI

recruitment of Tag and/or pol-α primase to replication origin and facilitate the assembly of pre-initiation complex (Figure 2.12). NFI activity can be modulated by TGF-β(4, 5), TNF-α(5), oxidative stress(9, 95-97), which are all changed in allograft kidneys (21, 40, 67).

The regulation of BKV replication by NFI-family transcription factors is complicated due to multiple NFI isotypes/splicing variants and multiple binding sites present on enhancer. In addition to the proposed mechanism, NFI might also regulate viral DNA replication through chromatin remodeling (30, 100) or indirectly through modulation of early gene expression(23). How BKV DNA replication and gene transcription is coordinated by NFI-family transcription factors during BKV reactivation in PVAN needs to be investigated in detail.

Another possible implication of our proposed model is that NFI might participate in cellular DNA replication by recruitment of pol-α primase to the cellular DNA replication origin. Around 30,000~50,000 origins are active during replication of mammalian DNA, however, unlike the prokaryotes DNA replication, the origins of mammalian genome do not have a consensus sequence and not all the origins are active at the same time(85). And the selection of origins is related to differentiation and development(85). Like well-established viral systems(101), transcription factor binding sites and promoters coincide with some eukaryotic cellular replication origins (75). And several evidences have shown that transcription factors do involve in the cellular replication (10, 17, 49, 131), perhaps through interaction with components of DNA replication machinery and/or chromatin remodeling in response to intracellular/extracellular signals during the development, differentiation and stress response (38, 75). The expression and activity of NFI-family transcription factors is greatly affected by development, differentiation and stress (9, 26, 77, 96, 97, 111, 120). So it is possible that NFI plays a role in the selection of cellular DNA replication origins during development/differentiation and in response to stress.

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CHAPTER 3

Acetylation of BKV large T antigen and modulation of BKV DNA replication by histone acetyltransferases (HATs)

INTRODUCTION

As with SV40 and mouse polyomavirus (mPyV), BK virus (BKV) core origin flanking sequences regulate BKV DNA replication (28, 46). Various mechanisms by which auxiliary *cis*-acting elements regulate viral DNA replication have been suggested (13, 32) including: recruitment of components of the DNA replication machinery; modulating chromatin structure; activating replication factors; preventing the binding of inhibitory factors; and facilitating the localization of template to replication factors in the nuclei. Chromatin remodeling through histone acetylation/deacetylation by HATs (histone acetyltransferases)/HDACs (histone deacetylases) is one of the likely mechanisms for how transcription factors can regulate viral DNA replication, as for transcription (23, 44). In support of this, SV40 minichromosomes replicate more efficiently *in vitro* when histones are hyperacetylated or when the acetylated histone N-termini are removed(1, 36).

In addition to histones, HATs and HDACs also target viral or other cellular proteins, to modulate their activities and functions. Thousands of acetylated cellular proteins have been identified using high-resolution mass spectrometry; and over fifty acetylated proteins are directly involved in DNA replication processes (9), many of which, including RPA, RFC, PCNA, topoisomerase I and DNA

polymerase δ, etc. are essential components for polyomavirus DNA replication (19). In addition to these replication factors, large T antigens (Tags) from SV40 and mPyV, are also modified by acetylation (35, 51). But the functional consequence of the acetylation of polyomavirus Tags remains unknown. Tag forms a double-hexamer in the presence of ATP and binds the replication origin to initiate DNA replication by melting DNA duplex in the origin, followed by unwinding of dsDNA for DNA elongation with its helicase/ATPase activity in the presence of ATP/Mg^{2+} (4, 17-19). Steps in these processes might be controlled by acetylation, just as DNA binding activity of Tag is regulated by phosphorylation (31, 39, 40).

We have previously shown that histone acetyltransfererases (HATs) PCAF/GCN5 acetylate mPyV Tag and stimulate mouse polyomavirus (mPyV) DNA replication when tethered to the core origin(51). The function of mPyV Tag acetylation has not been established. Others have observed that SV40 Tag is acetylated by CBP/p300 at K697 in the C-terminal host range (HR) domain mediated through interaction with p53(3, 35, 41), and this acetylated lysine is conserved among Tags of SV40, BKV and JCV(35). But none of the newly identified human polyomaviruses (KI, WU, Merkel) have this conserved lysine(2, 20, 21), due to the lack of HR domain. Although acetylation of SV40 Tag K697 has no effect on the host range phenotype of SV40 (34), acetylation was suggested to regulate protein turnover of Tag (41).

Here we demonstrate that PCAF/GCN5 acetylate both BKV and SV40 Tags; the acetylation site of BKV Tag is K687, which is conserved with the CBP/P300 acetylation site (K697) in SV40 Tag. Furthermore, acetylation of BKV Tag appears to somewhat regulate its stability, as previously reported for SV40 Tag. However, in contrast to the stimulation of mPyV DNA replication observed with ectopic expression of PCAF/GCN5, BKV and SV40 replication are strongly inhibited by ectopic expression of PCAF/GCN5; furthermore, mutation of K687 in BKV Tag has no effect upon the inhibition of replication by ectopic expression of PCAF/GCN5, indicating inhibition of BKV DNA replication by PCAF/GCN5 is not caused by acetylation of Tag. Instead, PCAF/GCN5 must

target other component(s) of the DNA replication machinery. A search for these targets is proposed, and possible functions of BKV and SV40 Tags' acetylation are discussed.

MATERIALS AND METHODS

Plasmids. Test templates are pBKGal4, pSV40Gal4. All mammalian expression vectors are in a same pCMV- backbone, which derives from pcDNA3.1 (Invitrogen) with deletion of the SV40 origin; mammalian expression vectors for Tags are pCMV-BKT-Flag, pCMV-SV40T; for HATs expression vectors are pCMV-GalDBD, pCMV-PCAF, pCMV-PCAF(Δ65-112), pCMV-PCAF(Δ573-608), pCMV-GCN5, pCMV-GCN5(DEY), pCMV-GCN5(FTE), pCMV-p300, which are cloned into the pCMV- backbone from their original expression vectors described in (51). The cDNAs of truncated fragments of BKV Tag and SV40 Tag were cloned into bacteria expression vectors pCDFDuet(Novagen) with PCR cloning.

Expression and purification of Tags

The full-length BKV Tag with Flag epitope tag was expressed using Bac-to-Bac Baculovirus Expression System (Invitrogen). $1.5X10^7$ of Hi-Five insect cells were seeded in 150mm flasks and cultured overnight at 27°C. Hi-Five cells were infected at MOI=10 with baculovirus encoding Tag-Flag produced in Sf9 cells. Infected Hi-Five cells were harvested 48 hours post-infection (P.I) and lysed in 1ml of 0.5% NP-40 Lysis Buffer (50mM Tris-Cl, pH7.5, 150mM NaCl, 5mM KCl, 1.0mM $MgCl_2$, 0.5% NP-40, 0.5mM DTT, 10% glycerol, 1X PhosSTOP phosphatase inhibitors (Roche), 1X Complete protease inhibitor cocktail (Roche)) by incubation on a rotating platform at 4°C for 30 minutes; then the lysate was transferred to a glass Dounce tissue grinder (Wheaton Scientific) and homogenized with 20 strokes using a type A pestle; the lysate was cleared by centrifugation at 12000rpm(20,000g) in a Sorvall SA-600 rotor for 30 minutes in 4°C; the supernatant was incubated with 60ul of Anti-Flag M2 Affinity Gel (Sigma) by rotating at 4°C for 2 hours; after incubation, the Anti-Flag M2 Affinity Gel was washed once with 0.6ml of ice-cold

Wash Buffer 1 (50mM Tris-Cl, pH7.5, 300mM NaCl), twice with 0.6ml of ice-cold Wash Buffer 2 (50mM Tris-Cl, pH7.5, 50mM NaCl) by rotating 5-10 minutes in 4°C. Tag was eluted twice with 0.1ml of ice-cold Elution Buffer A (50mM Tris-Cl, pH7.5, 150mM NaCl, 5mM KCl, 1mM MaCl2, 0.01% NP-40) supplemented with 250mg/ml Flag peptide by rotating at 4°C for 20 minutes. The two elutions were pooled and dialyzed in dialysis buffer (20mM HEPES, pH8.0, 150mM NaCl, 1mM DTT, 10% glycerol).

Various truncated His- tagged BKV Tags and C-terminal domain of SV40 Tag were expressed in Rossetta^{TM} 2 *E.coli* cells (Novagen), a BL21 strain carrying tRNAs for rare codons to enhance expression of eukaryotic genes in bacteria. Transformed Rossetta^{TM} 2 cells were cultured in 250ml LB medium supplemented with 34ug/ml chloramphenicol and 40ug/ml spectinomycin in a shaking platform at 225rpm in 25°C. Expression of the fusion proteins was induced with 1mM isopropyl-1-thio-D-galactopyranoside (IPTG) when the density of *E.coli* cells reached 0.4-0.5 (OD_{600}). After induction, *E.coli* cells were cultured in 25°C with 225rpm shaking for 20 hours. To purify the proteins, *E. coli* cells were lysed by sonication (twice, 300 seconds each, 20% duty cycle, maximum power) in 30ml Buffer L2 (50 mM Tris-Cl, pH 8.0, 250 mM NaCl, and 1 mM β-Mercaptoethanol, 10% glycerol, 1mM PMSF, Complete protease inhibitor cocktail (Roche)). 0.1% NP-40 was added into the lysates after sonication and incubated on ice for 10 minutes. After separating the soluble supernatant of the cell lysates from the pellet by centrifugation at 12,000rpm(20,000g) in Sorvall SA-600 rotor for 30 minutes at 4°C, the fusion proteins in the supernatant fraction were incubated with 0.4ml TALON Metal Affinity Resin (Clontech) at 4°C for 2 hours followed by washing the beads three times with 5ml of Buffer L2 supplemented with 1mM PMSF and 5mM imidazole by rotating at 4°C for 5 minutes. Truncated Tag was eluted with 1ml of Buffer L2 supplemented with 150mM imidazole, followed by a second elution with 1ml of Buffer L2 supplement with 250mM imidazole with rotating at 4°C for 5 minutes. Concentrations of purified Tags were determined with the Bradford Protein Assay.

***In vitro* acetylation assays**

Acetylation reactions for Tags were performed with a mixture of 5ug of baculovirus-expressed full-length Tag or 5ug of bacteria-expressed truncated Tags with 1-2ug PCAF(Upstate) or GCN5(BIOMOL) or CBP (BIOMOL) or p300 (BIOMOL) and 4uM(200pmol) acetyl-coenzyme A (Sigma) in 50ul of HAT assay buffer (50 mM Tris-Cl pH 8.0, 0.1 mM EDTA, 1.0 mM dithiothreitol (DTT), 10% glycerol) at 30°C for 1 h. Reaction mixtures were stopped by addition of 2X SDS sampling buffer. Acetylation was detected by Western blotting by using rabbit anti-acetyl lysine polyclonal antibody (Cell Signaling).

Acetylation reactions for synthetic peptide (Biomatik) were performed with a mixture of 0.5ug of peptide with 2ug PCAF(Upstate) and 4uM(200pmol) acetyl-coenzyme A (Sigma) in 50ul of HAT assay buffer (50 mM Tris-Cl pH 8.0, 10% glycerol) at 30°C for 1 h. The acetylated products were analyzed with MALDI (Matrix-assisted laser desorption/ionization)/TOF-TOF mass-spectrometry (MU Proteomic Core).

***In vivo* acetylation assays**

$2.2X10^6$ HEK293 cells in 60mm plate were transfected with expression vectors for Tag (1.9ug) and HATs (1.9ug). 18 hours post-transfection, cells were treated 0.1ug/ml Trichostatin A (TSA) for 4 hours; then cells were harvested and lysed with 200ul of 1% Triton Lysis Buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), Complete protease inhibitor cocktail (Roche), 1X PhosSTOP phosphatase inhibitors (Roche)) by rotating for 1 h at 4°C; and lysates were cleared by centrifugation at 12000rpm(20,000g) in Sorvall SA-600 rotor for 30 minutes at 4°C. The supernatants were incubated with 50ul of Anti-Flag M2 Affinity Gel (Sigma) or 50ul of Anti-HA Affinity Matrix (Roche) by rotating at 4°C for 2 hours, followed by two washes with 0.5ml of the 1% Triton Lysis Buffer. The pelleted beads were suspended in 40ul of 1X SDS sample buffer, boiled for 5 minutes. Samples of 20ul of the

supernatant were loaded for SDS-PAGE and Western Blotting analysis using rabbit anti-acetyl lysine polyclonal antibody (Cell Signaling).

In vivo DNA replication assays

HK-2 cells were seeded in 12-wells plates ($1X10^5$ cells/well), and incubated overnight at 37°C. Cells were transiently tranfected with 1.0ug total DNA (0.2ug template + 0.04ug pCMV-BKT-Flag + 0.4ug pCMV-HATs (unless indicated) + 0.36ug pUC-empty) and 3ul FugeneHD transfection reagent (Roche) in 1000ul RPMI medium 1640 supplemented with 10% fetal bovine serum (Hyclone), 4 mM L-glutamine without antibiotics. Similarly, human HEK 293 cells and CV-1 were seeded in 12-well plates ($4X\ 10^5$ cells/well and $2X10^5$ cells/well, respectively), and incubated overnight at 37°C. Both HEK293 and CV-1 cells were transfected with 0.7ug total DNA (0.15ug template + 0.05ug pCMV-BKT-Flag + 0.5ug pCMV-HAT) with 2ul LipofectAMINE and 5ul PLUS reagent (Invitrogen). After incubating cells with a DNA: LipofectAMINE and PLUS mixture for 4 to 5 h in 500ul of serum-free DMEM, the transfection solution was replaced with 1 ml of DMEM containing 10% FBS. Cells were harvested at 48 h P.T (post-transfection). And low-molecular-weight (LMW) DNAs were extracted following the Hirt protocol(25) and purified with Promega Miniprep columns. The purified LMW DNAs were digested with EcoRI to linearize the plasmid, and digested with DpnI to distinguish input from replicated DNA in Tango Buffer (Fermentas). The DpnI- resistant DNA was resolved from digested DNA by agarose gel electrophoresis (1%). After capillary transfer of the DNA to a nylon membrane, DpnI-resistant DNA was detected by Southern blotting with a biotinylated probe of the BKV core origin (~80 nucleotides) and visualized by chemiluminescent nucleic acid detection (Pierce).

RESULTS

PCAF/GCN5 acetylate BKV Tag *in vivo* and *in vitro.*

To determine if PCAF acetylates BKV Tag, Flag tagged full-length BKV Tag was expressed in the baculovirus expression system, purified using Flag affinity chromatography (see Materials and Methods) and incubated in an *in vitro* acetylation reaction system containing acetyl-CoA and purified recombinant PCAF. Acetylated Tag was detected by Western Blotting using anti-acetyl-lysine (anti-AcK) antibody: a band at size of BKV Tag (90kD) was detected when Tag was incubated with PCAF (Figure 3.1A, lane 2), and no acetylation of Tag was observed in the absence of PCAF (Figure 3.1A, lane 2). Although PCAF strongly autoacetylates, there was no acetylation signal in lanes loaded with PCAF alone at the position corresponding to the size of BKV Tag (Figure 3.1A, lane3). This experiment indicates that PCAF acetylates BKV Tag *in vitro*.

In vivo Acetylation of BKV Tag in by PCAF and GCN5 also was detected. Expression vector for Flag-tagged BKV Tag was transiently co-transfected with HATs (Figure 3.1B) into HEK293 cells. Transfected cells were treated with TSA for 4 hours at 20h post-transfection (P.T). After immunoprecipitation from cell extracts using Flag antibody, acetylated Tag was detected with Western Blotting using anti-AcK antibody. Analogous to what was observed in the *in vitro* acetylation assays, PCAF strongly acetylated BKV Tag *in vivo* (Figure 3.1C, lane 1), while PCAF mutant (Δ573-608) with a deletion in the HAT domain did not acetylate Tag (Figure 3.1C, lane 3) and a mutant PCAF (Δ65-112) with deletion in CBP/p300 interaction domain acetylated BKV Tag at a reduced level (Figure 3.1C, lane 2), suggesting this domain is not required for acetylation, but may play a regulatory role. GCN5 also acetylates BKV Tag *in vivo* (Figure 3.1C, lane 5); two GCN5 mutants with point mutations in the HAT domain behave differently: the FTE mutant is completely defective in acetylation of BKV Tag (Figure 3.1C, lane 7); but the DEY mutant acetylates BKV Tag stronger than wildtype GCN5, despite that Tag expression is expressed at reduced level (Figure 3.1C, lane 6). This indicate that DEY mutation in the HAT domain does not disrupt the HAT activity of GCN5, but might have increased its acetylation activity for BKV Tag; acetylation of Tag may reduce the Tag expression by decreasing its protein stability. Surprisingly, CBP/p300, which acetylate SV40 Tag *in vivo* (35), do not, if at all, acetylate BKV Tag in HEK293 cells (Figure 3.1, lane 8-9; very weak bands of corresponding to proteins the size

of BKV Tag were detected after extended exposure of film (data not shown)). So PCAF/GCN5, but not CBP/p300, acetylate BKV Tag *in vivo*.

Figure 3.1 Acetylation of BKV Tag *in vitro* and *in vivo*. (A). In vitro acetylation assays. Purified full-length BKV Tag was acetylated in vitro by PCAF. Lane 1, Tag without PCAF; lane 2, Tag with PCAF; lane 3, PCAF without Tag. Acetylated signal was detected with anti-aceytlK antibody. The signal from acetylated Tag and PCAF is indicated with arrow and solid line respectively. Input of Tag was detected with coomassie staining.

Figure 3.1A

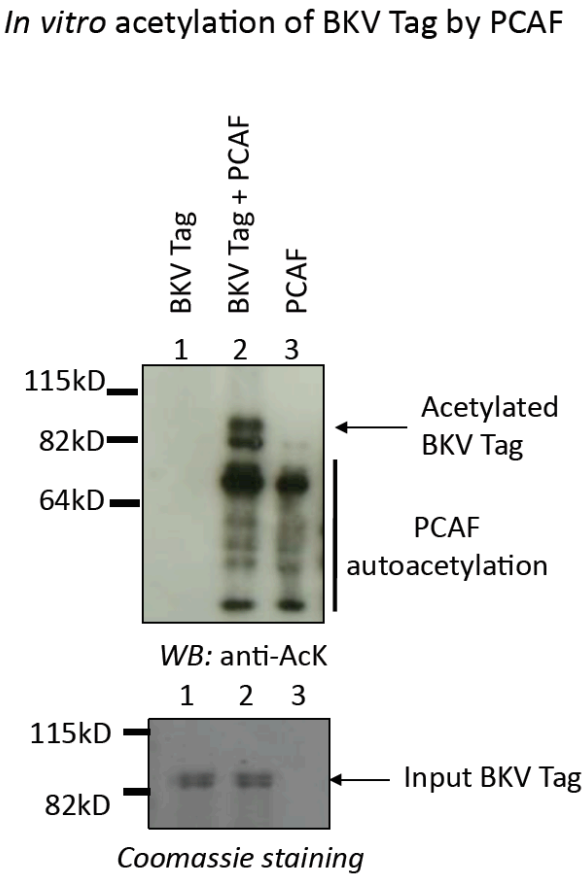


Figure 3.1 (B). Structures of histone acetyltransferases (HATs). HAT domains are indicated by light bars. Two HAT substitution mutants of small form (SF) human GCN5 (FTE and DEY), one HAT deletion mutant of PCAF (574-608), and one deletion mutant of PCAF in the p300/CBP interacting domain (65-112) also are indicated. (C). In vivo acetylation assays. Tag was co-transfected with different HATs into HEK293 cells. Acetylation of Tag in vivo was detected by Western Blotting using anti-AcK antibody. Input of Tag was detected using anti-Flag antibody.

Figure 3.1B

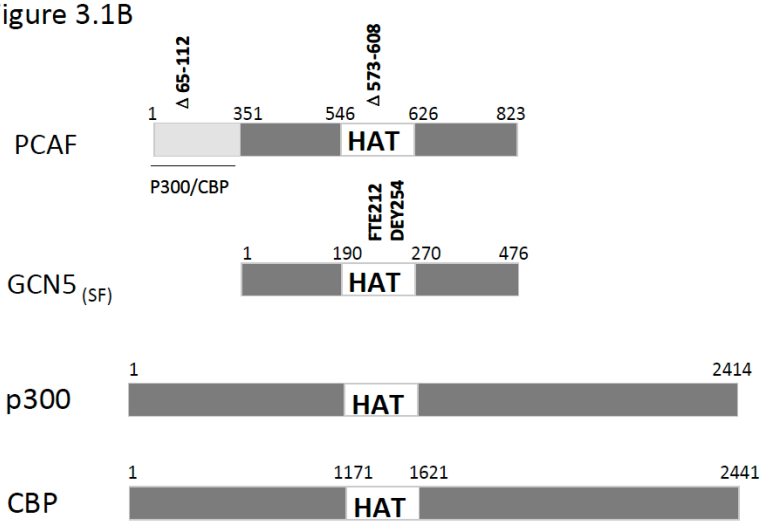
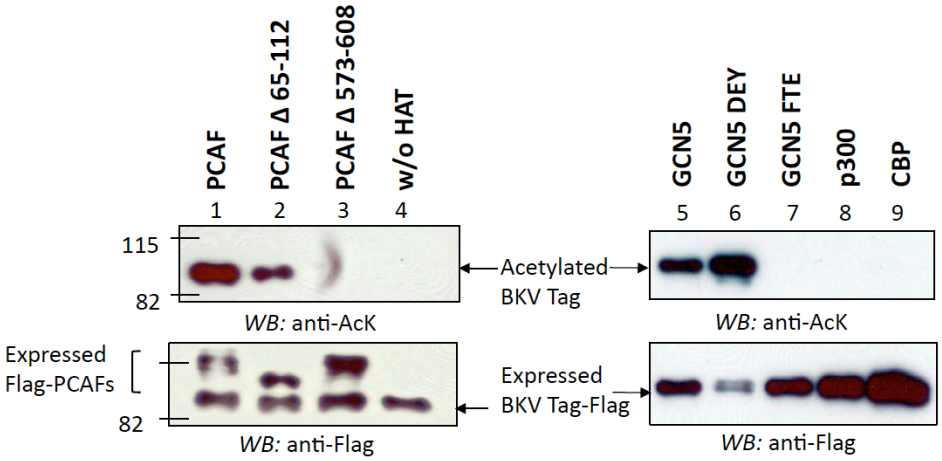


Figure 3.1C

In vivo acetylation of BKV Tag by PCAF



BKV Tag C-terminal HR domain is acetylated

Several truncated His tagged BKV Tag proteins (Figure 3.2A) were expressed in *E.coli* and purified over TALON resin™. Acetylation of these truncated Tags was tested in the *in vitro* acetylation assays. Two truncated BKV Tags containing HR domain were acetylated by PCAF (Figure 3.2B, lane 2, 3), while the mutant lacking the HR domain was not (Figure 3.2B, lane 1). This indicates that HR domain of BKV Tag is acetylated, similar with SV40 Tag (35, 41).

To compare the acetylation of BKV and SV40 Tag by different HATs, the same truncated C-terminal HR domain (Cter) from both Tags were expressed in *E.coli* and purified. The acetylation by GCN5 and CBP/ p300 was tested in the *in vitro* acetylation assays. GCN5, but not CBP/p300, strongly acetylates BKV Tag (Figure 3.2C, lane 2-4). This is consistent with the observation *in vivo* that PCAF/GCN5 acetylates BKV Tag much better than CBP/p300 (Figure 3.1C). SV40 Tag is acetylated by GCN5 and CBP/p300 (Figure 3.2C, lane 6-8), consistent with previous reports that SV40 Tag is acetylated by CBP/p300 (35, 41).

Figure 3.2 *In vitro* acetylation of truncated Tag. (A). Illustration of full-length and truncated BKV Tag. (B). In vitro acetylation of truncated BKV Tag by PCAF; the position of acetylated Tag was indicated by arrow.

Figure 3.2A

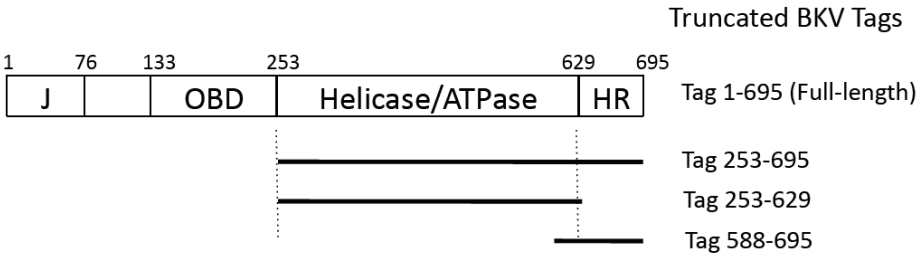


Figure 3.2B

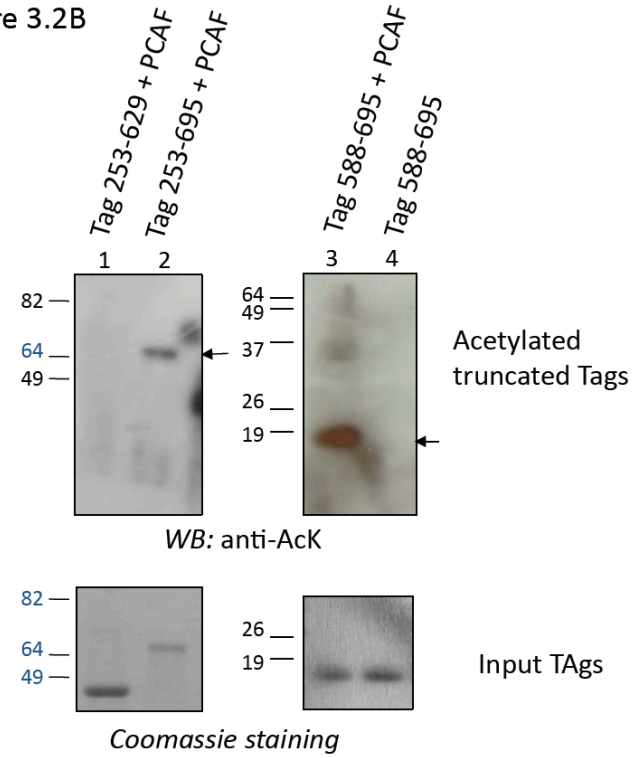
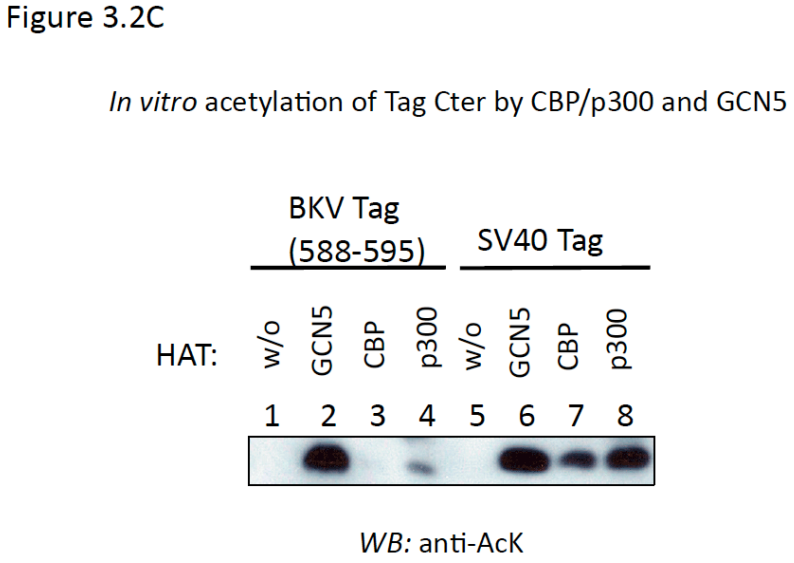


Figure 3.2 (C) In vitro acetylation of BKV and SV40 Tags C-terminal truncated fragments by various HATs. Lane 1-4, Cter of BKV Tag; lane 5-8, Cter of SV40 Tag. The HAT used in the acetylation assay was indicated above each lane; w/o stands for without HAT. Acetylated truncated Tag was detected by anti-AcK.



K687 is the only acetylation site in BKV Tag

There are four lysines in the HR domain of BKV Tag, which are all located in the most C-terminal end (681~695aa). To determine which lysine(s) is/are acetylated by PCAF/GCN5, a synthetic peptide with amino acid sequence from BKV Tag 687-695aa was acetylated by PCAF *in vitro*, then analyzed by MALDI-TOF/TOF mass-spectrometry. The MALDI-MS analysis of the peptide after acetylation shows two strong peaks with a mass difference of 42Da (Figure 3.3A), the characteristic mass shift for a acetyl group (15). No further +42Da was observed, indicating that only one lysine is acetylated. *De novo* sequencing of the acetylated peptide by tandem MS/MS analysis indicates that K687 is acetylated (Figure 3.3B). The mass of analyzed peptide was 2Da lower than the theoretical value, due to the disulfide formed between cysteine residues 680 and 685 (Figure 3.3B). Inclusion of DTT in the *in vitro* acetylation assay confirmed this (data not shown).

To determine whether K687 is the only acetylation site in BKV Tag, the K687 residue of BKV Tag was mutated to Arginine (R). Truncated C-terminal BKV Tag (588-695aa) with the HR domain was tested in the *in vitro* acetylation assays (Figure 3.3C). While wildtype BKV Tag (588-695) was acetylated by GCN5 efficiently (Figure 3.3C, lane1), no acetylation of the Tag K687R mutant was detected with either GCN5 or CBP (Figure 3.3C, lane 2-3). And acetylation of BKV Tag *in vivo* by PCAF was also completely disrupted by K687R mutation (Figure 3.3D, lane 1-3). These data indicate that K687 is the only acetylation site in BKV Tag.

Figure 3.3 Determining acetylation site of BKV Tag. (A). Analysis of acetylated BKV Tag C-terminal peptide (681-695aa) by MALDI-TOF mass spectrometry. The peaks from non-acetylated and acetylated peptide are indicated by arrow. The acetylation of peptide causes 42Dalton shift from non-acetylated peptide.

Figure 3.3A

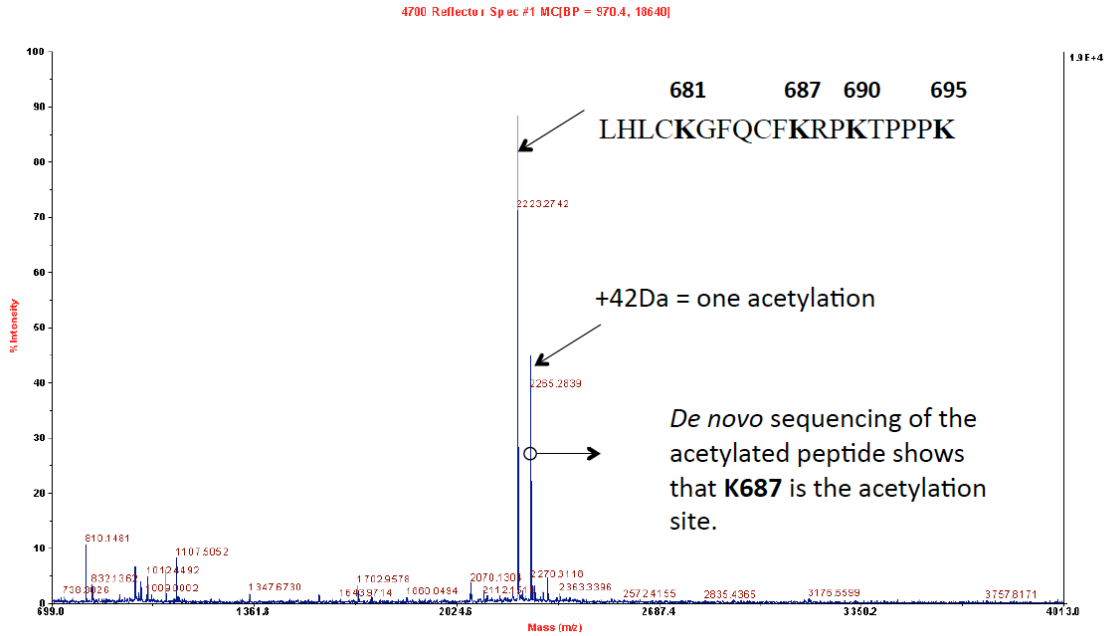


Figure 3.3 (B). *De novo* sequencing of the acetylated peptide by tandem MS/MS analysis. Identification of ions fragmented at different orientations is indicated by different colors: Red is from C- to N-terminus; Blue is from N- to C-terminus. Locations of acetylated lysine and the disulfide bond between two cysteine residues are also indicated.

Figure 3.3B

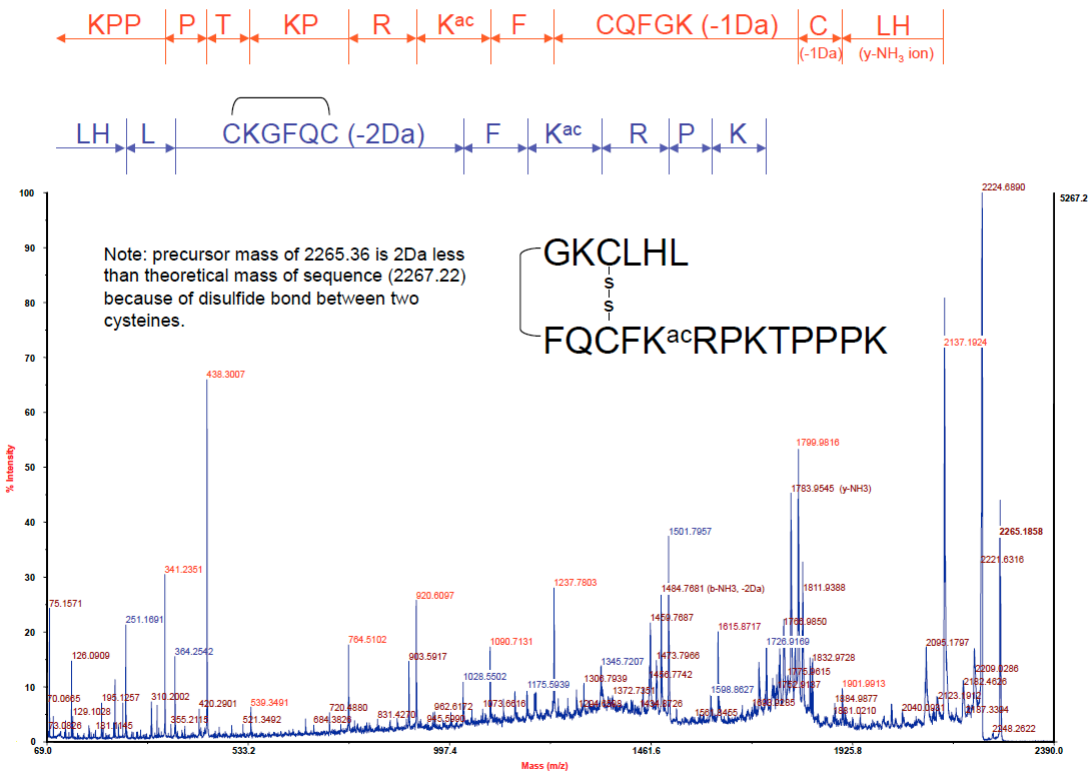


Figure 3.3 (C). In vitro acetylation of wildtype and mutant Cter of BKV Tag (588-695). Lane 1, acetylation of wildtype BKV Tag by GCN5; lane 2, acetylation of K687R mutant BKV Tag by GCN5; lane 3, acetylation of K687R mutant BKV Tag by CBP. (D). In vivo acetylation of full-length wildtype and mutant BKV Tag by PCAF. Lane 1, acetylation of wildtype BKV Tag by PCAF; lane 2, acetylation of K687R mutant BKV Tag by PCAF; lane 3, acetylation reaction in the absence of Tag.

Figure 3.3C

In vitro acetylation of BKV Tag (588-695)

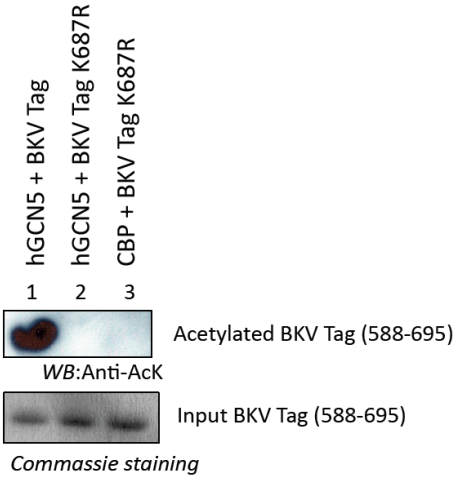
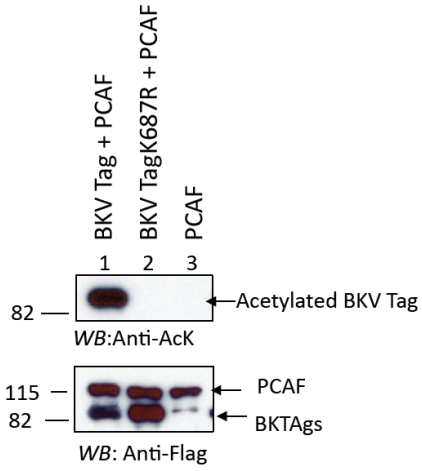


Figure 3.3D

In vivo acetylation of BKV Tag



Expression of Gal4-PCAF/GCN5, but not Gal4-CBP/p300 inhibit BKV and SV40 DNA replication when tethered to the core origin.

To determine whether acetylation of Tag regulates BKV/SV40 DNA replication, expression vectors for Gal4 fusion HATs (Figure 3.1B) and BKV/SV40 templates with 5XGal4 binding sites (Figure 3.1A) were co-transfected with cognate Tag expression vectors into HEK293/HK-2 cells (for BKV) or CV-1 cells (for SV40). The 5XGal4-binding sites replacing the enhancer region of SV40 and BKV resemble the recruitment of HATs to their replication origins by transcription factors/co-factors.

Surprisingly, in contrast to mPyV (51), replication of templates with either BKV or SV40 origins was strongly inhibited by Gal4-PCAF/GCN5, compared with Gal4 without fused HAT (Figure 3.4B, lane 1-2, 6; Figure 3.4C, lane 1-2, 5; Figure 3.4D, lane 1-3). The inhibition by Gal4-PCAF/GCN5 is dose-dependent on input level of Gal4-GCN5 (Figure 3.4B, lane 2-5; Figure 3.4C, lane 1-4). However, no inhibition of BKV replication was observed with expression of Gal4-CBP/p300 (Figure 3.4B, lane 7; Figure 3.4C, lane 6); and strong stimulation of SV40 replication was observed with expression of Gal4-p300 (Figure 3.4D, lane 4).

To determine if the inhibition of BKV DNA replication by PCAF/GCN5 is dependent on acetylation, replication of BKV DNA by wildtype and K687R mutant BKV Tags was compared in HK-2 cells. PCAF/GCN5 co-expression with the K687R mutant Tag inhibits DNA replication (Figure 3.4E, lane 6-9) as it does with wildtype Tag (Figure 3.4E, lane 1-4), suggesting that inhibition of BKV DNA replication by PCAF/GCN5 coexpression is not determined by acetylation of Tag. The acetylation must affect some other function of Tag, such as its stability (41) or regulation of gene transcription or cellular transformation.

To test whether over-expression of PCAF/GCN5 change the expression of Tag, which could indirectly affect the BKV DNA replication, expression of BKV Tag in HK-2 cells was tested upon

co-expression of PCAF/GCN5. Increased expression of Tag was observed upon co-expression of PCAF/GCN5(Figure 3.4F). So PCAF/GCN5 must target other cellular proteins to inhibit the BKV DNA replication.

Figure 3.4 DNA replication assays with Gal-4 fusion HATs (A). Structure of Gal4 test templates for BKV and SV40. The enhancer region of BKV and SV40 NCCR is replaced by 5XGal4 binding sites. (B). Replication of pBKVGal4 template in HK-2 cells transiently expressing Gal4-fusion HATs. Arrow indicates the replicated template; star indicates DpnI digested input.

Figure 3.4A

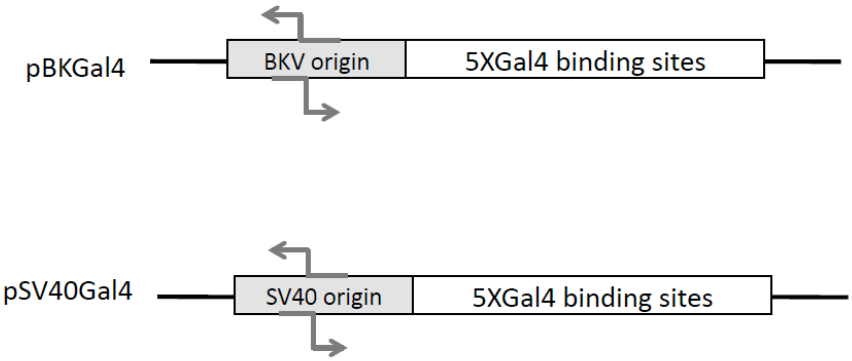


Figure 3.4B

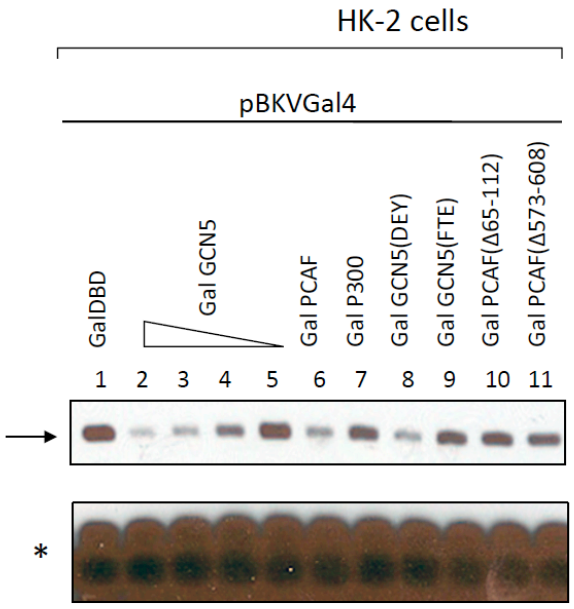


Figure 3.4 (C) Replication of pBKVGal4 template in HEK293 cells transiently expressing Gal4-fusion HATs. (D). Replication of pSV40Gal4 template in CV-1 cells transiently expressing Gal4-fusion HATs. (E). Replication of pBKVGal4 template by either wildtype or K687R mutant BKV Tag in HK-2 cells. (F). Expression of Tag with co-expression of HATs.

Figure 3.4C

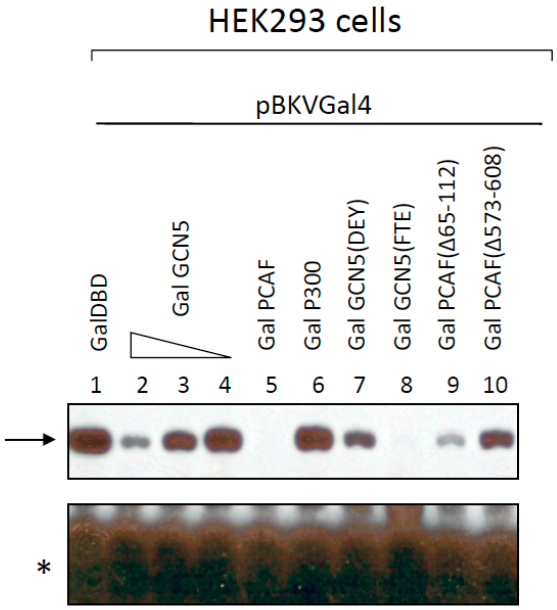


Figure 3.4D

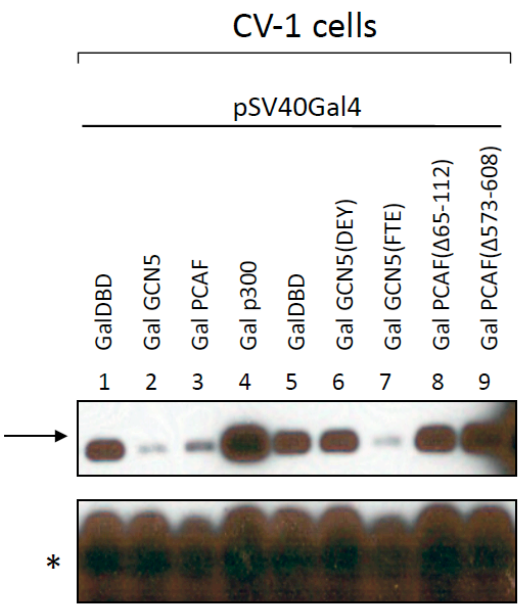


Figure 3.4E

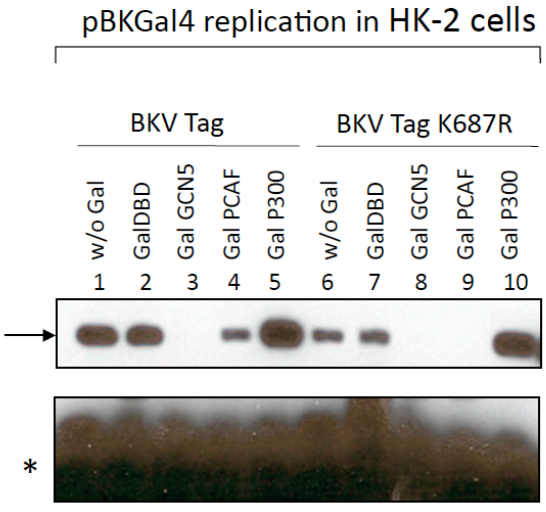
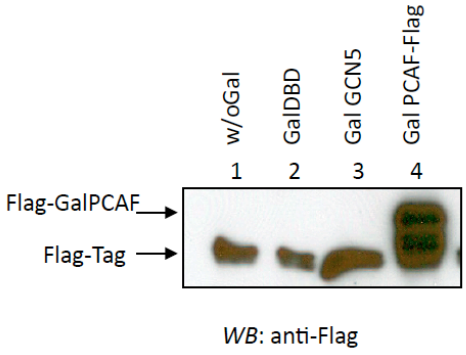


Figure 3.4F



Acetylation of BKV Tag regulates its protein stability

Although the acetylation of K687 in BKV Tag is not responsible for the inhibition of BKV DNA replication by PCAF/GCN5, the mutation of K687R reduced BKV DNA replication compared with wildtype Tag(Figure 3.4E). To examine whether enzymatic activities important for replication are altered by acetylation, the helicase activity of mutant and wildtype BKV Tag were compared with *in vitro* helicase assays, but no significant difference was observed (data not shown). Acetylation of SV40 Tag has been shown to modulate its protein stability(41). It is possible that K687R mutant BKV Tag is less stable than with wildtype Tag. The stability of wildtype and mutant BKV Tag was compared in HEK293 cells with CHX chase assays (Figure 3.5). The transient acetylation of Tag (Figure 3.5, lane 5-8) and K687R mutation (Figure 3.5, lane 9-12) reduced the stability of T antigen, compared with wildtype Tag without transient acetylation by PCAF (Figure 3.5, lane 1-4). So both acetylation and the K687R mutation decrease the protein stability of BKV Tag. This is different from the report that acetylation of SV40 Tag decrease the protein stability, while K697R mutation stabilize the protein(41). However, our result is consistent with the replication results showing that BKV DNA replication is less efficient with K687R mutant Tag than wildtype Tag. The mechanism of how this acetylation regulates stability of Tag is unclear; but it seems not to be regulated through proteasome-degradation pathway(41).

In conclusion, inhibition of BKV DNA replication by PCAF/GCN5 is not caused by the acetylation of Tag by PCAF/GCN5; although acetylation of Tag may influence its protein stability, it is not responsible for the inhibition of BKV DNA replication by PCAF/GCN5. PCAF/GCN5 must act through other cellular replication factors to modulate BKV DNA replication.

Figure 3.5 CHX chase assays to determine the protein stability of BKV Tag affected by acetylation. (A). Expression of Tag was detected by Western Blotting. Lane 1-4, Tag was co-expressed with HAT mutant PCAF in HEK293 cells; lane 5-8, Tag was co-expressed with wildtype PCAF in HEK293 cells treated TSA; lane 9-12, Tag K687R mutant was co-expressed HAT mutant PCAF in HEK293 cells. Time of harvest post-CHX treatment was indicated above each lane. β-actin was used as loading control. (B). Quantification of results from Western Blotting. The density of each band from Tag was quantified and normalized with relevant β-actin density.

Figure 3.5A

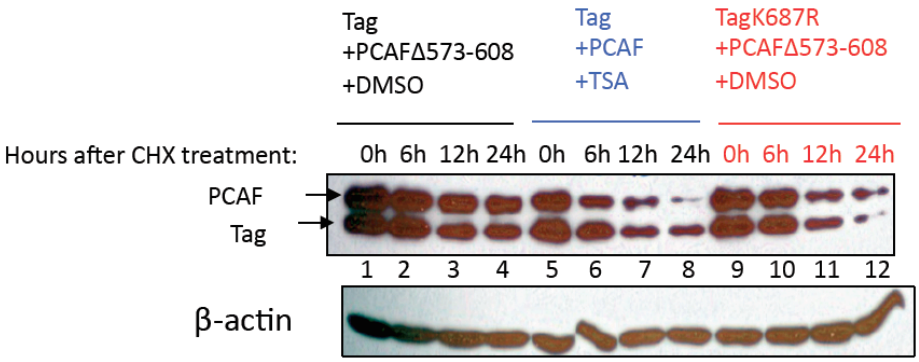
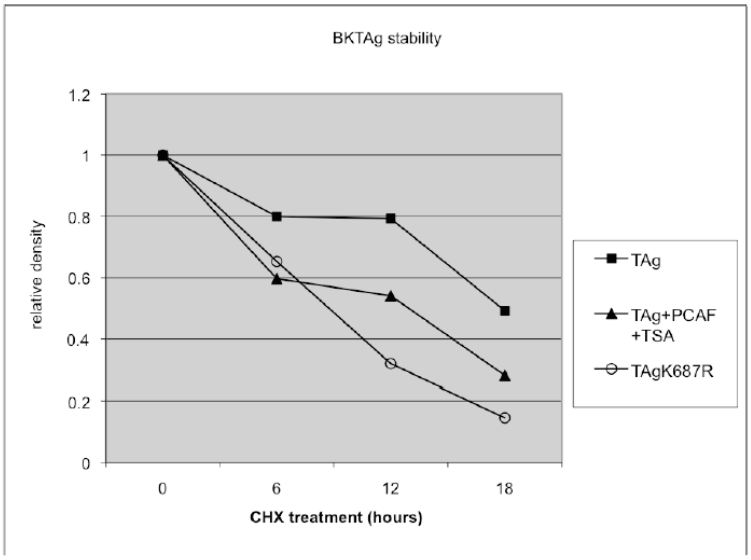


Figure 3.5B



DISCUSSION

Replication of polyomavirus genome is initiated from the core origin and regulated by core origin flanking sequences, to which transcription factors/co-factors bind (12, 14, 32). The chromatin configuration of polyomavirus minichromosomes around the noncoding control region (NCCR) determines the activity of viral transcription and replication (1, 7, 8, 11, 22, 50). Acetylation of histones by HATs is an important mechanism of chromatin remodeling and regulation of gene transcription and DNA replication (1, 23, 36, 37, 44). Besides histones, HATs also acetylate other cellular or viral proteins to modulate their activities, some of which are known to affect polyomavirus DNA replication directly, these including SV40/mPyV Tags(35, 51), p53(24, 27, 38), Sp1(45), c-Jun(49), pRb(6), E2F(29, 30).

Here, we found that BKV Tag is acetylated *in vitro* and *in vivo* by PCAF/GCN5 at K687, a conserved site of Tags from three primate polyomavirues, BKV, JCV and SV40, which however is not present in newly identified KI, WU and Merkel Cell human polyomaviruses(2, 20, 21). CBP/p300 acetylates SV40 Tag (K697) *in vivo* through interactions with p53 (3, 35). And the interaction with p53 is indispensible for acetylation of SV40 Tag *in vivo* (35). However, we found CBP/p300 can acetylate SV40 Tag efficiently in the absence of p53 *in vitro* (Figure 3.2C), suggesting that p53 is not absolutely required for acetylation. Instead, it promotes the acetylation of SV40 Tag by facilitating the interaction between CBP/p300 and Tag *in vivo*(3). However, no stable complex between BKV Tag and CBP/p300 or PCAF/GCN5 has been detected in HEK293 cells, while stable interaction between BKV Tag and p53 can be detected (data not shown). This might be because the p53 in HEK293 cells is not phosphorylated at S15, which enhances the interaction of p53 with CBP/p300 (3, 16, 26).

Despite the homology in their HR domains, BKV and SV40 Tags are acetylated with different efficiencies by PCAF/GCN5 and CBP/p300. PCAF/GCN5 acetylates BKV Tag much more efficiently than CBP/p300 *in vivo* (Figure 3.1C) and *in vitro* (Figure 3.2C, lane 1-4). SV40 Tag can

be acetylated by either PCAF/GCN5 or CBP/p300 *in vitro* (Figure 3.2, lane 5-8). This is consistent with the observation that CBP/p300 acetylates SV40 Tag *in vivo* (3, 35, 41). Whether PCAF/GCN5 acetylate SV40 Tag *in vivo* is unknown. Although down-regulating endogenous PCAF by siRNA did not affect SV40 Tag acetylation *in vivo* (35), this might be due to low expression of endogenous PCAF or to the high expression of endogenous GCN5 and CBP/p300 as redundant sources for acetylation. So in conclusion, PCAF/GCN5 can efficiently acetylate SV40 and BKV Tags, while CBP/p300 only selectively acetylate SV40 Tag, but not BKV Tag. The slight difference of amino acid sequence in the HR domains of SV40 and BKV Tag might determine their different susceptibility for acetylation by different HATs.

PCAF/GCN5, but not CBP/p300, inhibited BKV DNA replication in HK-2 and HEK293 cells when tethered to the origin (Figure 3.4B lane 1-11; Figure 3.4C). A similar inhibitory effect of PCAF/GCN5 was also observed with SV40 DNA replication in CV-1 cells (Figure 3.4D, lane 1-9). This is in contrast to the stimulation of mPyV replication in NIH3T3 and FOP cells by PCAF/GCN5 (51). K687R mutation did not prevent the inhibition by PCAF/GCN5 (Figure 3.4E, lane 6-9) suggests that PCAF/GCN5 targeting other cellular protein(s) to inhibit BKV DNA replication. The acetylation site of mPyV Tag has recently been mapped to the second nuclear localization signal (NLS-2) in the variable domain of mPyV Tag (Olga Kenzior and Folk unpublished data). Interestingly, mutation of this lysine also did not abrogate the stimulation of mPyV DNA replication by ectopic expression of PCAF/GCN5 (Olga Kenzior and Folk unpublished data). The distinct effects of PCAF/GCN5 on polyomavirus DNA replication in primate cells and murine cells appears not to be determined by acetylation of Tags. The basic DNA replication machineries of murine and primate polyomavirueses are highly conserved. Then, what could be the reason for this difference? One most likely possibility is that components of DNA replication machinery or other related factors in primate and murine cells might be differently modified by PCAF/GCN5 and have different consequences on DNA replication. Another more complicated possibility is that PCAF/GCN5 inhibits the same replication factor(s) both in murine cells and primate cells; but because PCAF/GCN5 stably interact with mPyV Tag and stimulate mPyV DNA replication by

recruiting Tag to origin of replication (51), which overcomes the inhibitory effect upon other factors, PCAF/GCN5 still stimulate mPyV DNA replication; however, because PCAF/GCN5 does not stably interact with BKV Tag, no stimulation of BKV DNA replication is observed. These hypotheses will be tested in future studies.

Although no stimulation of BKV DNA replication by PCAF/GCN5 was observed in this study, the possibility that HAT modulates chromatin structure of polyomavirus to promote DNA replication cannot be excluded. The Gal-p300 strongly stimulates SV40 in CV-1 cells (Figure 3.4D lane 1, 4) and perhaps weakly stimulates BKV DNA replication in HK-2 cells (Figure 3.4E lane 1, 5, 6, 10) when tethered to the origin of replication, while no effect was observed with mPyV (51). This suggests that p300 and PCAF/GC5 might affect polyomavirus DNA replication through different mechanism. More detailed study is needed to determine the stimulatory mechanism of p300 for SV40 and perhaps BKV DNA replication.

Acetylation of SV40 and mPyV Tags has been reported (35, 41, 51), but the function of Tag acetylation still remains elusive. The acetylation site of BKV and SV40 Tags is located in the highly conserved HR domain, which is the last 26 amino acids at the C-terminal end (as characterized in SV40 Tag) (48). Different strains of SV40 isolated from monkey and human tissues have several mutations and deletions in the HR domain; however, K697 is not affected, suggesting this acetylation site might be essential for viral replication (5). Although some mutations are close to the K697 (5), whether they affect the acetylation of K697 is unclear. HR truncation mutant of SV40 fails to grow in CV-1 cells (33, 47, 48) and displays a temperature sensitive phonotype in permissive BSC cells(10, 33). The failure of HR deletion mutant SV40 to grow in nonpermissive CV-1 cells or at nonpermissive temperature in BSC cells can be rescued by providing by C-terminal HR domain *in trans* (34, 48). Some studies indicate that the function of HR domain might be related with control of late gene transcription(43) and virion assembly(42). However, mutation of the SV40 Tag acetylation site does not affect the host range phenotype (34), suggesting the acetylation is not important for the host range activity. We and others have

found that acetylation of SV40 and BKV Tags might regulate their protein stability(41) (Figure 3.5A, B), but the mechanism is unclear and need to be tested with more sensitive assays, such as GPS (Global Protein Stability assay)(52).

In conclusion, BKV and SV40 Tags are acetylated specifically at a conserved lysine residue in the HR domain by CBP/p300 or PCAF/GCN5. The acetylation appears to be not essential for viral proliferation in cell culture, probably because the HAT's expression and activity are stable and constant in the homogeneous cultured cells. The effect of acetylation might be more important *in vivo* for the initial acute infection, spreading of virus through the body, establishment of persistent infection and reactivation, which needs to be tested in animal models.

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CHAPTER 4

Restriction of BKV DNA replication in murine cells and inhibition of BKV DNA replication by noncoding RNAs

INTRODUCTION

BKV and JCV polyomaviruses are endemic in humans but in certain individuals can cause nephropathy and leukoencephalopathy, respectively, and also may be associated with some cancers and other pathologies (2, 5, 16-18, 27, 29, 49). Recently, additional human polyomaviruses have been discovered (KI, WU and the Merkel cell polyomavirus (MKV)(1, 13, 15), and preliminary evidence indicates the first two are associated with the oral cavity and respiratory system and the third is associated with and perhaps the cause of Merkel cell carcinomas (reviewed by (8, 21, 22)). Remarkably, despite the considerable understanding of the biology of the *Polyomaviridae*, there is little understanding of how these viruses establish and maintain persistent infections, nor are there good means to abrogate or mitigate the infections and pathologies caused by these viruses.

Replication of polyomavirus DNA requires the large T antigen (Tag), with the host supplying the other replication factors (12, 19, 28, 46). Polyomavirus Non-Coding Control Regions (NCCRs) comprise the origins of DNA replication and *cis*-acting regulatory elements both 5' and 3' of the core origin (12, 19, 28) that bind Tag and cell factors regulating viral transcription and DNA replication (9, 31). The development of *in vitro* DNA replication systems has allowed the

identification and characterization of the proteins required for polyomavirus replication (26, 28, 30, 34): the Tag helicase activity unwinds DNA in an ATP- and phosphorylation-dependent process (11, 12, 28, 33, 35, 38, 48), and single-stranded DNA (ssDNA) is bound by replication protein A (RPA), and topoisomerase I relieves torsional stress ahead of the replication fork (3, 40). Subsequently the primase activity of DNA polymerase α-primase (Pol-α primase) synthesizes short RNA primers that are elongated by DNA polymerase α (10). Leading strand synthesis is completed by DNA polymerase δ, RPA, proliferating nuclear antigen (PCNA) and replication factor C (RFC). Replication of the lagging strand is mediated by RPA, Pol-α primase, DNA polymerase δ and auxiliary proteins (35, 38, 39, 48). Heretofore, there has been no hint that small cellular RNAs might also regulate polyomavirus DNA replication through the viral NCCRs, although recently it has been determined that small ncRNAs may directly regulate eukaryotic DNA replication (24), and that RNAs help to recruit the cellular origin recognition complex (ORC) to the Epstein Barr Virus (EBV) origin of replication and stimulate EBV DNA replication (37).

SV40 (simian virus 40) does not replicate in mouse cells nor do mouse cell extracts support their *in vitro* replication, and in turn, mouse polyomavirus (mPyV) does not replicate in human cells or human cell extracts (6, 23, 42-44). Restriction of SV40 replication occurs at the level of initiation by Pol-α primase (6, 44, 46). But why BKV replication is restricted in murine cells has not been investigated.

Our analysis of the restriction of BKV replication in murine cells revealed a more complex regulation: while the human Pol-α primase complex is required for BKV DNA replication, other factors within mouse cells and extracts inhibit replication (28, 46). Inhibitory activities were purified, some of which were determined to be small cellular RNAs, termed small replication regulatory RNAs (srRNAs), that act through the BKV NCCR. These suggest that cellular small noncoding RNAs regulate viral DNA replication, which may have great implication in viral tropism, establishment of persistent infection and reactivation.

MATERIALS AND METHODS

Plasmids. pOriBKV (termed B-B-B in Figure 4.1A, Figure 4.2A) was generated by inserting PCR fragments of whole NCCR (positions 5031 to 282) of archetype BKV Dik strain (kindly provided by J. Lednicky; GenBank Accession #AB211369) into the polylinker region(XmaI/PstI) of pUC18 plasmid. Other similar pUC18-based plasmid DNAs with complete viral origins included pOriJCV (Mad-1 strain), pOriSV40 (SV-S strain), and pOrimPyV (P-P-P; A3 strain). Chimearic templates were generated by ligation of PCR fragments into XmaI/PstI sites of pUC18 plasmid. The pUC18 plasmid without an insert served as negative control (pOri-) for cell-free DNA replication as well as a vector for cloning all viral origins.DNAs for replication assays were verified by sequencing and purified with Qiagen Midiprep kits. pU6 was constructed by insertion of a U6 promoter DNA fragment cut out with Bgl II and EcoR I from a modified pSirenRetreQ vector into the BamH I and EcoR I digested pUC18 vector. pU6-B-5-1 and pU6-mY1 were constructed by insertion of annealed synthetic oligonucleotides of DNA encoding B-5-1 and mY1 RNA sequences into pU6 vectors using BamH I and EcoR I sites. Sequences of oligonucleotides

for B-5-1 are

5’GATCACCGGTCTCACGACGACATTCCAGACGTGGCCTCGTGGGTGCTTCCACGTTGCGAA CACCCCGATTTCCCGGTCCCTTTTTTGGAA 3’ and

5’AATTTTCCAAAAAAGGGACCGGGAAATCGGGGTGTTCGCAACGTGGAAGCACCCACGAGG CCACGTCTGGAATGTCGTCGTGAGACCGGT3’;

for mY1 are

5’GATCGGCTGGTCCGAAGGTAGTGAGTTATCTCAATTGATTGTTCACAGTCAGTTACAGATT GAACTCCTGTTCTACACTTTCCCCCCTTCTCACTACTGCACTTGACTAGTCTTTTTTGGAA 3’ and

5’AATTTTCCAAAAAAGACTAGTCAAGTGCAGTAGTGAGAAGGGGGGAAAGTGTAGAACAGG AGTTCAATCTGTAACTGACTGTGAACAATCAATTGAGATAACTCACTACCTTCGGACCAGCC 3’.

In vivo DNA replication assays. Murine TCMK-1 cells were grown in DMEM with 10% FBS, seeded in eight-well plates (1 X 105 cells/well), and incubated overnight at 37°C. Cells were transfected with Lipofectamine and Plus reagent (Invitrogen, Carlsbad, CA) with expression vectors for TAg (0.6 ug of DNA) and template plasmid (0.4 ug). After incubation of cells with DNA-Lipofectamine and PLUS reagent mixture for 4 h in 500 ul serum-free DMEM, the transfection solution was replaced with 2 ml of DMEM containing 20% FBS. Similarly, human HEK 293 cells were grown in DMEM with 10% FBS, seeded in 12-well plates (4 X105 cells/well), and incubated overnight at 37°C. Cells were transfected with expression vector for TAg (5 ng), template plasmid (50 ng), and pUC18 empty vector (0.65 ug) as carrier DNA with Lipofectamine and Plus reagent. DNA Lipofectamine and Plus reagent mixtures were incubated with HEK 293 cells as described above. Human kidney proximal tubular epithelial cells (HK-2 cells) were grown in RPMI-1640 with 10% FBS, seeded in 12-well plates (1 x 105 cells/well), and incubated overnight at 37°C. Cells were transfected with expression vector for TAg (10 ng), template plasmid pOriBKV (10 ng), srRNA expression vectors pU6-mY1/pU6-B-5-1 or empty control vector pUC (200 ng), and pBC plasmid vector (500 ng) as carrier DNA using Fugene HD (Roche) transfection reagent. Cells were harvested at 48 h after transfection, and low-molecular-weight DNAs were isolated by the Hirt protocol with Promega Miniprep columns, digested with EcoRI to linearize the plasmid, and digested with DpnI to distinguish input from replicated DNA. The DpnI resistant DNA was resolved from digested DNA by agarose gel electrophoresis (1%). After transfer of the DNA to a nylon membrane, DpnI-resistant DNA was detected by Southern blotting with a biotinylated probe of the lacZ gene (~400 nucleotides) of the pUC18 vector and visualized by chemiluminescent nucleic acid detection (Pierce).

In vitro DNA replication assays. Replication of DNAs in vitro was assayed as described by Stadlbauer and coworkers with slight modifications (94). Briefly, the reaction mixtures (30 ul) contained 20 mM HEPES (pH 7.8); 7 mM magnesium acetate; 1 mM DTT; 4 mM ATP; 200 uM each CTP, UTP, and GTP; 50 uM dCTP; 100 uM each dATP, dTTP, and dGTP; 40 mM creatine

phosphate di-Tris (pH 7.8); 40 ug/ml creatine kinase plus 5 %Ci of [α32P]dCTP (3,000 Ci/mmol); 0.25 ug test plasmid DNA; FM3A or HeLa cell extract (25 to 75 ug of protein); and purified TAgs at the indicated concentrations. After incubation for 60 min at 37°C, reaction products were precipitated with cold 10% (wt/vol) trichloroacetic acid containing 2.5% (wt/vol) sodium pyrophosphate, spotted on glass fiber filters (GF/C; Whatman), washed with 1 M HCl, and analyzed by scintillation counting.

Monopolymerase DNA replication assay. The BKV and SV40 monopolymerase replication assay contained 0.25 µg of pOriBKV DNA or 0.5 µg of pOriSV40, respectively. The assay was supplemented with 50 ng topoisomerase I, 100 ng Pol-primase and 1 µg RPA, in 30 mM HEPES-KOH, pH 7.8, 7 mM MgAc, 0.1 mM EGTA, 0.5 mM DTT, 200 µM each UTP, GTP, and CTP, 4 mM ATP, 100 µM each dATP, dGTP, and dTTP, and 10µM dCTP, 40 mM creatine phosphate, 1 µg creatine kinase, 0.1 mg/ml heat-treated BSA, and 5 µCi [α32P]-dCTP (3000 Ci/mmol, Perkin-Elmer) in 40 µl. The small RNAs were added to the assay as indicated in the figure legends. Purified BKV or SV40 TAg (0.2 µg) was added to start the reaction as indicated, and after incubation for 60 min at 37°C, the reaction products were precipitated with cold 10% (w/v) TCA containing 2.5% (w/v) sodium pyrophosphate and spotted on glass fiber filters (GF/C; Whatman), washed with 1 M HCl and analyzed by scintillation counting.

RESULTS

Restriction of BKV DNA replication in murine cells.

BKV infection of murine cells suggested that BKV DNA replication is restricted in murine cells (data not shown)(28). To determine whether BKV NCCR is responsible for the restriction, replication of chimearic BKV templates with BKV core origin and flanking sequences from either BKV or mPyV were analyzed in human and murine cells (Figure 4.1A). Substitution of BKV core

origin flanking sequences with those of mPyV reduced BKV replication efficiency in human cells (Figure 4.1A lane1-4). These data indicate core origin flanking sequences regulate BKV DNA replication. However, chimearic templates with the BKV core origin do not replicate in murine cells (Figure 4.1A, lane 5-8), while templates with the mPyV core origin replicate well (Figure 4.1A, lane 9) (data not shown). BKV and mPyV Tags were equally expressed in murine cells (Figure 4.1B). These data indicate that while core origin flanking sequences modulate BKV DNA replication, the core origin is primarily responsible for the restriction of BKV replication in murine cells.

Figure 4.1 In vivo DNA replication assays of chimearic templates (done by Bo Liang) (A) In vivo DNA replication of BKV and mPyV in human and mouse cells. Vectors expressing BKV TAg (lanes 1 to 8) or mPyV TAg (lane 9) were cotransfected into human HEK293 (lanes 1 to 4) or mouse TCMK-1 (lanes 5 to 9) cells together with plasmids containing the complete BKV origin (lanes 1 and 5, B-B-B), the complete mPyV origin (lane 9, P-P-P), and BKV-mPyV chimeric origins (lanes 2 to 4 and 6 to 8). At 48 h after transfection, DNA was isolated and analyzed by Southern blotting. DNA replication products are marked by arrows. (B). Expression of BKV and mPyV Tags in transfected TCMK-1 cells, detected with anti-Flag antibody in Western Blot. Equal amount of crude cell lysates were loaded for each sample.

Figure 4.1A

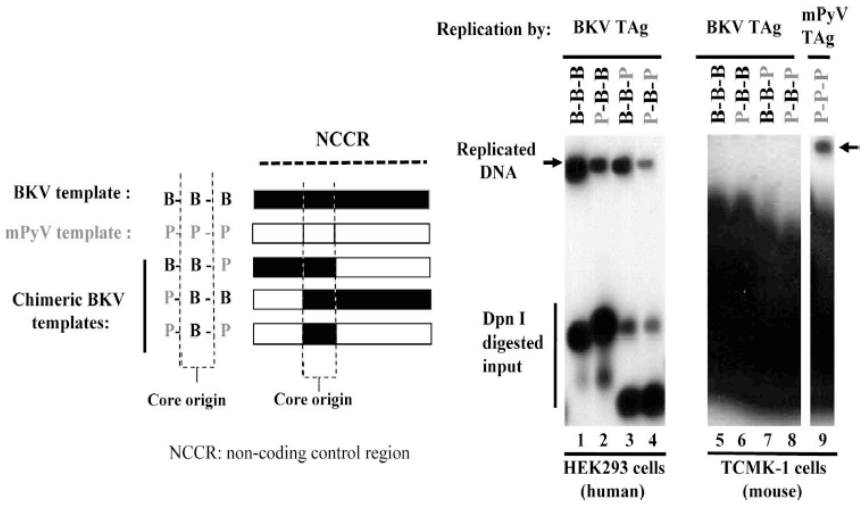
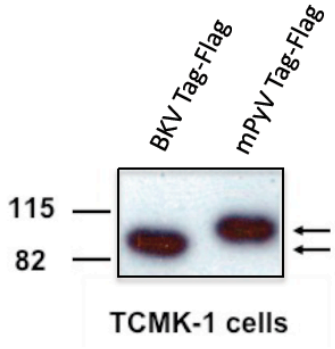


Figure 4.1B



WB: *anti*-Flag

Inhibition of BKV DNA replication by murine nuclear extracts.

In vitro DNA replication system was established by Mahon *et al*., with which restriction of BKV DNA replication in murine cells is reconstituted and studied *in vitro* (28). In murine FM3A nuclear extracts, neither BKV nor SV40 (Figure 4.2A, bars 2,3) replicate with cognate Tags; while mPyV replicates (Figure 4.2A, bars 1,4) with mPyV Tag. Adding human pol α-primase (polymerase α primase) into FM3A nuclear extracts allowed replication of SV40, but not BKV (Figure 4.2A, bars 5-7). This indicates that mechanisms for the restriction of BKV and SV40 DNA replication in murine cells are different. Adding either human RPA (hRPA) or Topoisomerase I (hTopo I) did not stimulate BKV DNA replication in FM3A nuclear extracts (Figure 4.2A, bars 9, 11), although hRPA and hTopoI are functional and can stimulate mPyV DNA replication in murine extracts (Figure 4.2A, bars 8, 10). This suggests that restriction of BKV DNA replication is not due to incompatibility between Tag and RPA or Topoisomerase I.

To determine whether functional interaction occurs between murine pol-α primase and BKV Tag, monopolymerase system containing purified components of initiation complex (pol-α primase, RPA, Topoisomerase I, Tag) was established. Using monopolymerase assays, Tikhanovich *et al.* found that murine pol-α primase does not support initiation of BKV DNA replication by BKV Tag (46). However, adding human pol-α primase into murine nuclear extracts does not support BKV DNA replication (Figure 4.2A, bars 5-7), suggesting in addition to pol-α primase, there is/are other inhibitory factors in murine nuclear extracts(46).

Mahon *et al.* also found that replication in the BKV monopolymerase system (BKV template and Tag, hRPA, hPol-α primase, hRPA) was not affected by human cell extracts added immediately prior to BKV TAg (Figure 4.2B, compare bars 1 and 2), whereas addition of murine extracts reduced the incorporation of dNMPs by almost 50% (compare bar 3 with bars 1 and 2)(28). In comparison, adding murine extracts reduced the incorporation of dNMPs in the SV40 monopolymerase system by only 10% (Figure 4.2C, compare bar 3 with bar 1), and the addition

of human extracts to the SV40 system did not influence replication (compare bars 1 and 2)(28). This indicates that some cellular factors in murine nuclear extracts inhibit the initiation of BKV DNA replication.

Figure 4.2 In vitro DNA replication assays (done by Cathal Mahon) (A). Modulation of polyomavirus DNA replication in murine cell extracts by human replication factors. In vitro DNA replication in the presence of equal amounts of purified recombinant BKV, mPyV, and SV40 TAg (shaded bars) using mouse FM3A cell extracts is shown. Bars 1 to 4 show DNA synthesis in the presence of mouse cell extracts and either mPyV TAg/P-P-P, BKV TAg/B-B-B, or SV40 Tag/pOriSV40. Incorporation of dNMPs into P-P-P in the absence of Tag served as a negative control. DNA synthesis with mouse cell extract and additional human DNA Pol-α primase (2 units of hPol-α primase) is depicted in bars 5 to 7 (mPyV TAg/P-P-P, BKV TAg/B-B-B, and SV40 TAg/pOriSV40, respectively). The DNA synthesis of mousecell extracts with an additional 0.5 %g of hRPA using mPyV TAg/P-P-P and BKV TAg/B-B-B is presented in bars 8 and 9, respectively, whereas the influence of human topoisomerase I (hTopo I, 120 ng) on the DNA synthesis in the presence of mouse cell extracts with mPyV TAg/P-P-P and BKV TAg/B-B-B is shown in bars 10 and 11, respectively. All assays were carried out in triplicate, and the results presented are the averages from two independent experiments. (B). The effect of human and mouse nuclear extracts on DNA synthesis by hPol-α primase was determined with a BKV origin of replication. The incorporation of radioactive dNMPs using the BKV origin of replication as a template was measured in the presence of buffer but no additional proteins or with 15 ug human or mouse cell extracts (bars 1, 2, and 3, respectively). DNA synthesis in the presence of DNA lacking an origin of replication served as a negative control (bar 4). (C) The effect of human and mouse proteins on the DNA synthesis by human DNA Pol-α primase was determined with an SV40 origin of replication. The incorporation of radioactive dNMPs was determined in the presence of buffer but no additional proteins or with 15 ug human or mouse cell extracts (bars 1, 2, and 3, respectively). DNA synthesis in the presence of DNA lacking an origin of replication served as a negative control (bar 4). Incorporation of dNMPs into DNA was measured by scintillation counting. DNA synthesis was determined in duplicate and repeated three times. The averages from these experiments and the standard deviations are presented.

Figure 4.2A

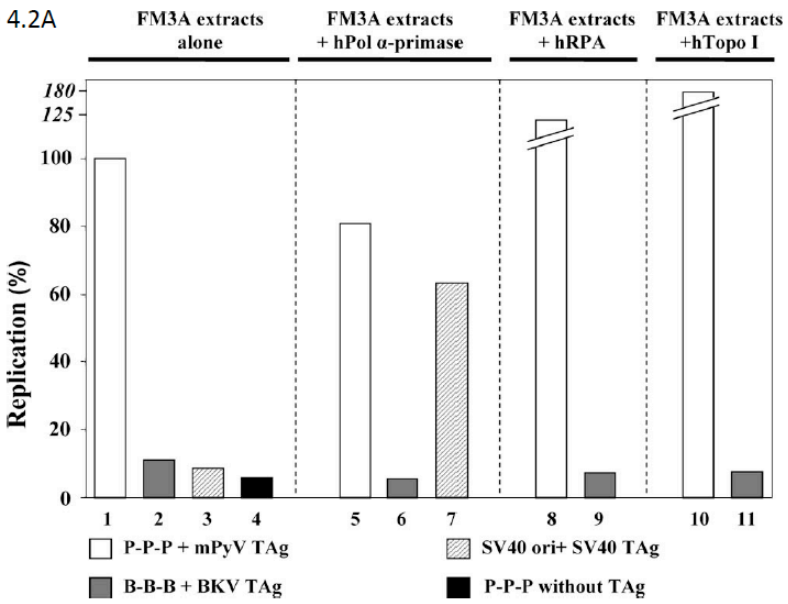


Figure 4.2B

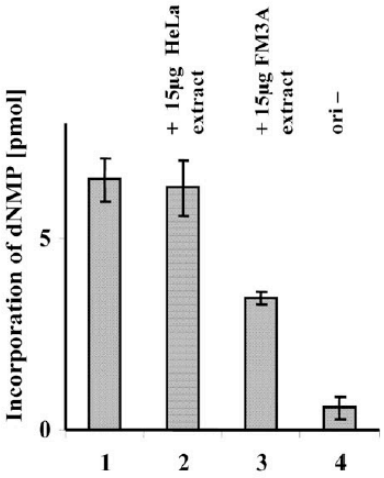
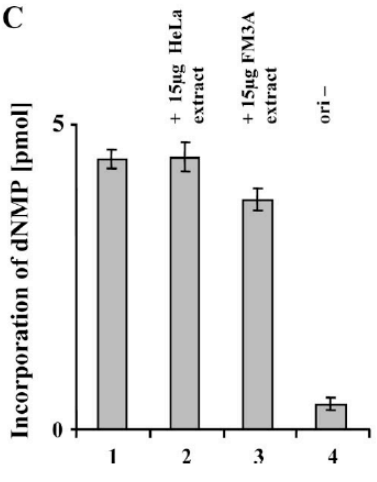


Figure 4.2C



Inhibitory activities for BKV replication in mouse cell extracts are small noncoding RNAs specific for the BKV NCCR.

To identify the inhibitory factors, mouse FM3A nuclear extracts were fractionated and inhibitory activity of each fraction was tested with monopolymerase assay by Tihkanovich *et al*.(45). Fractions containing nucleic acids with a size of less than 100 nt correlated with inhibitory activity (data not shown). RNase treatment but not DNase treatment of the fractions with maximal inhibitory activity abolished inhibition of BKV DNA replication *in vitro* (Figure 4.3A) indicating the IA is composed of small RNAs, hereafter called srRNAs(45).

To assess the specificity of the fractionated srRNAs, the highly purified small RNA fractions were added to monopolymerase replication assays with either BKV or SV40 TAg and DNA containing their cognate NCCRs. These analyses done by Tihkanovich *et al.* revealed that the inhibition of BKV replication by srRNAs is specific for the BKV NCCR and is independent of the source of TAg (the SV40 TAg can act at the BKV origin (28); Figure 4.3B)(45). Notably, the full replication activity of the monopolymerase assay with the SV40 template indicates these srRNAs neither inhibit enzymatic activities of cellular replication factors nor those of TAg.

Figure 4.3 Determination of nature of inhibitory activity (done by Irina Tikhanovich) (A). Mono Q chromatography-purified IA from mouse FM3A cells was treated with RNase or DNase, and then tested in BKV monopolymerase replication assays. (B). srRNA fraction purified from FM3A extracts were added to the monopolymerase replication assay using DNA containing the BKV origin of replication with SV40 TAg, or DNA containing the SV40 origin of replication with BKV TAg (75 ng of RNA with an estimated length of 50 nt (length estimation of RNA is derived from the cloning results without SELEX) yield a 50% inhibition with an 36-fold molar excess of RNA over origin DNA). Reactions with BKV TAg and DNA containing the BKV origin of replication served as controls. The incorporation of dNMPs into the BKV origin DNA was detected by acid precipitation and scintillation counting. DNA synthesis was determined in duplicates and repeated three times. The average of incorporation of dNMPs and the standard deviations are presented.

Figure 4.3A

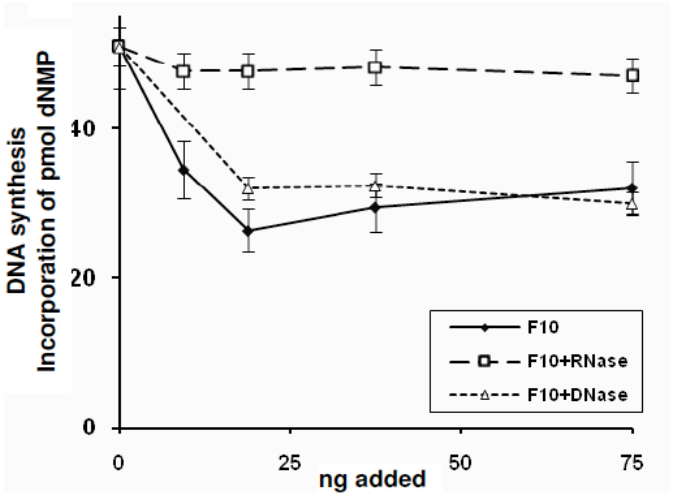
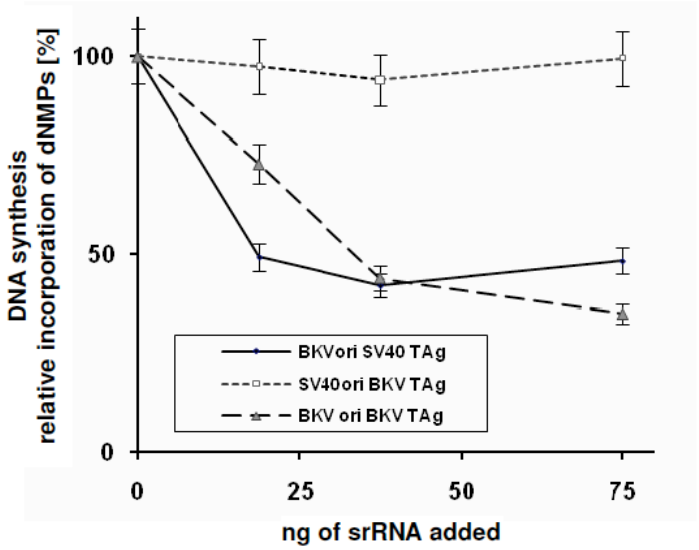


Figure 4.3B



Specific srRNAs inhibit BKV DNA replication.

Previous observations that templates containing BKV core origins with heterologous murine polyomavirus (mPyV) origin flanking sequences can be replicated by FM3A mouse cell extracts supplemented with human Pol-primase complex (46) and that inhibitory srRNAs are specific for BKV NCCR suggest that the inhibitory activity of srRNAs act through the viral NCCR. Specific srRNAs that can hybridize with denatured BKV NCCR were enriched and cloned with modified SELEX (Systematic Evolution of Ligands by EXponential enrichment). cDNAs derived from purified srRNAs were cloned and transcribed *in vitro*. Their inhibitory activities were tested in the *in vitro* DNA replication systems. The SELEX-selected B-5-1 and B-5-8 srRNAs inhibited *in vitro* BKV DNA replication to a greater extent than other RNAs (Figure 4.4A). These works are done by Irina Tikhanovich(45).

To ascertain if srRNAs function *in vivo*, the sequence of B-5-1 srRNA was placed under the U6 promoter (pU6-B-5-1). The pU6-B-5-1 was transiently transfected with BKV template and Tag expression vector into human kidney tubular epithelial cells (HK-2 cells). In multiple independent assays, BKV template replication in cells transiently expressing B-5-1 srRNA was consistently inhibited by up to 70% compared to transfections with the vector DNA (pUC) or the vector expressing an unspecific mouse Y-RNA (pU6-mY1) (Figure 4.4B), indicating srRNAs that inhibit the BKV replication *in vitro* also inhibit BKV DNA replication *in vivo*.

Bioinformatic analysis of B-5-1 and B-5-8 sequences indicates that they are complimentary to both strands of BKV NCCR; and mutations in the complimentary region disrupting the complimentarity also concomitantly abolish the inhibitory effect of srRNAs (data not shown) (45). *De novo* design of artificially srRNAs hybridizing with both strands of BKV NCCR also confirm that small noncoding RNAs complimentary to both strands of BKV NCCR can inhibit BKV DNA replication (45).

Figure 4.4 Effect of srRNAs on BKV DNA replication *in vitro* and *in vivo* (A) (done by Irina Tikhanovich) Replication of polyomavirus DNA in the presence of srRNAs. srRNAs were transcribed in vitro using oligonucleotides with an SP6 promoter; the sequence coding for the indicated RNAs as templates were assayed for inhibition of BKV DNA replication either in HeLa extract or in the monopolymerase replication assay. DNA synthesis was determined in duplicates and repeated three times. The average of incorporation of dNMPs and the standard deviations are presented. (B) (done by Bo Liang) In vivo BKV DNA replication in the presence of srRNA. BKV DNA replication in vivo was measured by transfecting HK-2 cells with BKV-origin containing plasmids and BKV TAg expression vector together with an empty vector (pUC, lane 1), or a control vector expressing mouse Y1-RNA (pU6-mY1, lane 2; mouse Y1-RNA also does not inhibit BKV DNA replication in vitro (Tikhanovich and Nasheuer, unpublished data)) or B-5-1 expression vector (pU6-B-5-1, lane 3). Cells were harvested 48 hours post transfection and replicated BKV DNA was analyzed by EcoR I and Dpn I digestion, agarose gel electrophoresis and Southern Blotting. The average quantification of band density of three in vivo DNA replication experiments and the standard deviation are presented. The amount of Dpn I-resistant replication products in the transfection of pUC empty vector were arbitrarily set to 100%.

Figure 4.4A

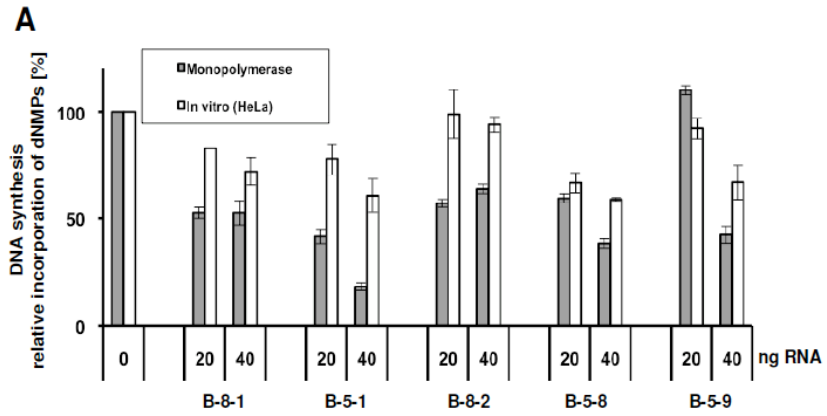
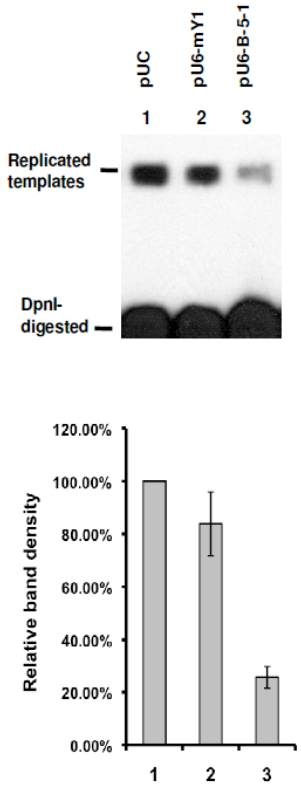


Figure 4.4B



DISCUSSION

BKV persistently infects most people, but how it establishes latency and reactivates in kidney allograft and causes PVAN in renal transplantation patients remains poorly defined. Immune-suppression is required but not sufficient for development of PVAN(14, 18, 32), suggesting other factors, such as ischemia/reperfusion injury, inflammatory response and tissue regeneration also contribute to the reactivation of BKV and pathogenesis of PVAN. Replication of polyomavirus is species-specific: like SV40 and JCV, BKV does not replicate in murine cells. Understanding the mechanism for the restriction of BKV replication in murine cells may shed light on how BKV maintains persistent infection and reactivates in kidney allograft. This may have implication for development of new anti-viral treatment for PVAN and other diseases caused by polyomavirus infection.

Infection of murine 3T3 cells with BKV suggests that viral DNA replication is restricted in murine cells and the restriction is not due to low expression of Tag (28). Over-expression of BKV Tag in murine cells dose not permit replication of BKV template in murine cells, further confirming that BKV DNA replication is blocked in murine cells (Figure 4.1A, B). Although the fundamental DNA replication machineries of different members of polyomaviruses are highly similar, the mechanisms for the host-specificity of their DNA replication seem to be slightly different: the host-specificity of mPyV and SV40 DNA replication is due to incompatibility of Tag with host pol-α primase (6, 23, 42-44); restriction of JCV DNA replication in murine cells appears to involve different mechanism(s), becausue murine pol-α primase supports *in vitro* JCV DNA replication in murine cell nuclear extracts (41); BKV Tag is incompatible with murine pol-α primase (46), however, adding human pol-α primase dose not support BKV DNA replication in murine extracts (Figure 4.2A), indicating restriction of BKV DNA replication in murine cells is not only due to incompatibility of Tag with murine pol-α primase, but also involves other inhibitory factor(s) present in murine cell nuclear extracts (Figure 4.2B,C).

Tikhanovich *et.al.* have fractionated the murine cell nuclear extracts and found that the fraction of small RNAs(~100nt) is mainly responsible for the inhibition of BKV DNA replication in an *in vitro* assay (Figure 4.3A)(45). Further characterization of these small RNAs (srRNA) indicates that some of them act through BKV NCCR and their inhibitory activity requires the hybridization of srRNAs with both strands of BKV NCCR(45). It has been proposed that the inhibitory srRNAs act through hybridization with both strands of BKV NCCR when the replication origin is opened up during the initiation of DNA replication. Which step(s) during the initiation is/are inhibited is not clear. RNA aptamers have been shown to inhibit SARS coronavirus helicase activity(20); but helicase activity of BKV Tag is marginally affected by srRNAs (data not shown)(45), which cannot account for their strong inhibitory effect on DNA replication. Enzymatic activity of pol-α primase is unlikely to be significantly affected, because srRNAs hardly affect SV40 DNA replication, which uses the same pol-α primase complex (data not shown)(45); however, we could not exclude the possibility that the *de novo* primer synthesis is blocked by srRNAs. More detailed biochemical analysis of origin structure during the initiation in the presence and absence of srRNAs is needed to resolve the mechanism of srRNAs' action upon BKV DNA replication, and perhaps other polyomaviruses.

Noncoding RNAs have been shown to be required for chromosomal DNA replication of mammalian cells through unknown mechanism (7, 25) and also help recruit Origin Recognition Complex (ORC) to replication origin of Epstein-Barr virus to facilitate initiation of DNA replication (36). Here we uncovered an inhibitory function of cellular encoded small noncoding RNAs against BKV DNA replication. Interestingly, in contrast to srRNAs in murine cells (FM3A), the similar srRNAs fraction from human tumor cells (HeLa) stimulates BKV DNA replication *in vitro* (data not shown), suggesting cell type specific expressions of srRNAs may have distinct effect in regulation of viral DNA replication. The sequences of some srRNAs are similar with some ncRNAs that are highly over-expressed in cancer cells and are induced by stress or during differentiation (4, 47). It is tempting to speculate that differential expression of srRNAs in host cells has implication in the

viral tropism and viral pathogenesis through regulation of viral DNA replication. Deep sequencing of small RNAs in infected cells is needed to test this hypothesis.

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CHAPTER 5

Proposed Model and Future Directions

A proposed model: ischemia/reperfusion injury stimulates BKV DNA replication in kidney tubular epithelial cells.

After initial infection, BKV establishes a life-long persistent infection in kidney tubular epithelial cells without causing clinical symptoms (13, 19, 23, 42). The immune system suppresses BKV replication(6, 15), however, it does not completely eradicate BKV from infected kidneys. Pathogenic reactivation of BKV occurs predominantly in patients following kidney transplantation, causing polyomavirus associated nephropathy (PVAN)(41), suggesting the relation of BKV reactivation with particular physiological condition in kidney allografts.

It has been suggested that ischemia/reperfusion injury may be related with BKV reactivation in kidney allograft (3, 20, 24, 25). This is strongly supported by the evidence that chemical and mechanical injuries of kidneys can stimulate mPyV reactivation in persistently infected mice(3) and that SV40 infection triggers host cellular DNA-damage response and SV40 co-opts the DNA damage/repair pathway to facilitate its own replication(16, 44, 49, 50). This prompted us to investigate whether BKV DNA replication is stimulated by stresses during the kidney transplantation. Ischemia/reperfusion is a major cause for kidney allograft injury during renal transplantation, thus we hypothesized that ischemia/reperfusion injury stimulates BKV DNA replication in kidney tubular epithelial cells.

Because hypoxia treated HK-2 cells closely resembles the epithelial pathophysiology of kidney from ischemia injury(29), we tested how hypoxia and hypoxia/re-oxygenation affect BKV DNA replication in HK-2 cells. HK-2 cells were transiently transfected with low amounts of BKV wildtype template and expression vector for Tag; the replication of BKV template in four conditions were compared: 1) normoxia (20%O2) for 48 hours; 2) normoxia for 24hours followed by hypoxia (1% O2) for 24hours; 3) hypoxia for 24hours followed by normoxia for 24hours; 4) hypoxia 48hours. The effectiveness of hypoxia treatment was confirmed by detection of increased expression of Hypoxia-Inducible Factor (HIF1α) (data not shown) and Vascular Epithelial Growth Factor (VEGF) (Figure 5.2) (29). The results of the DNA replication assays indicate that hypoxia inhibits BKV DNA replication (Figure 5.1A, panel 1, 2, 4), but after re-oxygenation BKV DNA replication is stimulated ~2 fold compared with replication in normoxia for 48hours (Figure 5.1A, panel 1, 3). Considering the shorter period of time for active replication after re-oxygenation (24 hours) compared with 48 hours of replication in normoxia condition, the rate of BKV DNA replication after re-oxygenation should be more than 2 fold higher than the replication rate in normoxia condition. Although expression of Tag is inhibited by hypoxia, Tag expression level after re-oxygenation is close to that in normoxia condition (Figure 5.1 B), suggesting the stimulation of BKV DNA replication is due to modulation of cellular factor(s) or change of Tag's activity, perhaps through post-translational modification.

Although the stimulation of BKV DNA replication by hypoxia/re-oxygenation observed from the preliminary results is not large (Figure 5.1A), it is significant and repeatable. Two major technical challenges may greatly reduce the effect of ischemia/reperfusion injury. One challenge is that archetype BKV is hard to grow in cell culture, making it difficult to study the PVAN in cell culture model. To get around this, we focus on the DNA replication of archetype BKV in cell culture; however, this strategy neglects the regulation of BKV replication at transcriptional level, which might also be regulated by ischemia/reperfusion injury. Another technical challenge is the limitation of the cell culture model that we use. Although the renal proximal tubular epithelial cell line (HK-2) and primary cells (RPTEC) have some value for study of BKV replication, these cells

are still different or cultured under completely different conditions from those *in vivo*. They have different differentiation status and potential, lack normal kidney functions, and have altered metabolism; and their immortalization could change their susceptibility to stress (34). Also, the

Figure 5.1 BKV DNA replication in HK-2 cells under hypoxia/reoxygenation conditions. (A) BKV DNA replication assays under normal and hypoxia conditions. Each panel has three repeats. Panel 1, normal oxygen condition (normoxia; N) for 48h; panel 2, normoxia for 24h followed by hypoxia (H) for 24h; panel 3, hypoxia for 24h followed by normoxia 24h; panel 4, hypoxia 48h. (B) Expression of Tag under normal and hypoxia conditions. Two repeats of each condition were examined in 7.5% SDS-PAGE gel followed by Western Blotting using anti-Flag; one sample of each repeat was separated in 6% SDS-PAGE gel for better resolution.

Figure 5.1A

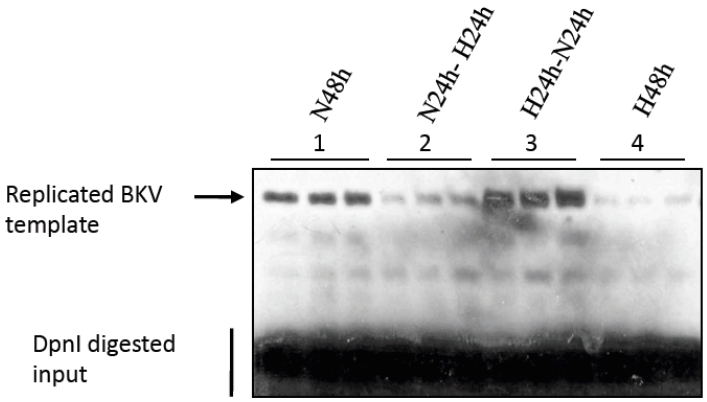
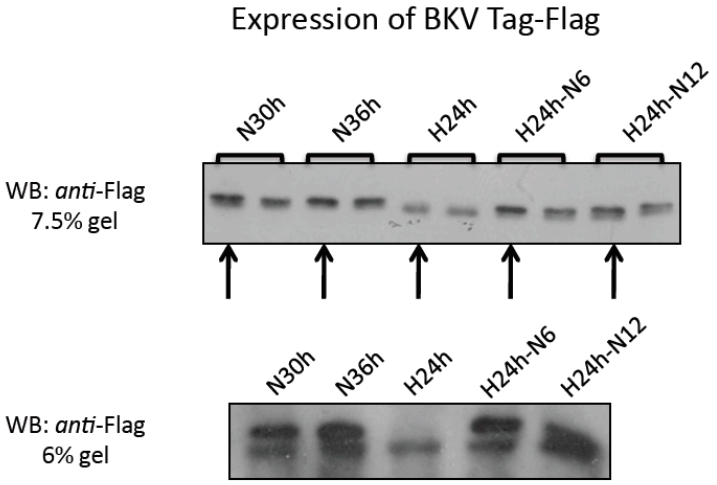


Figure 5.1B



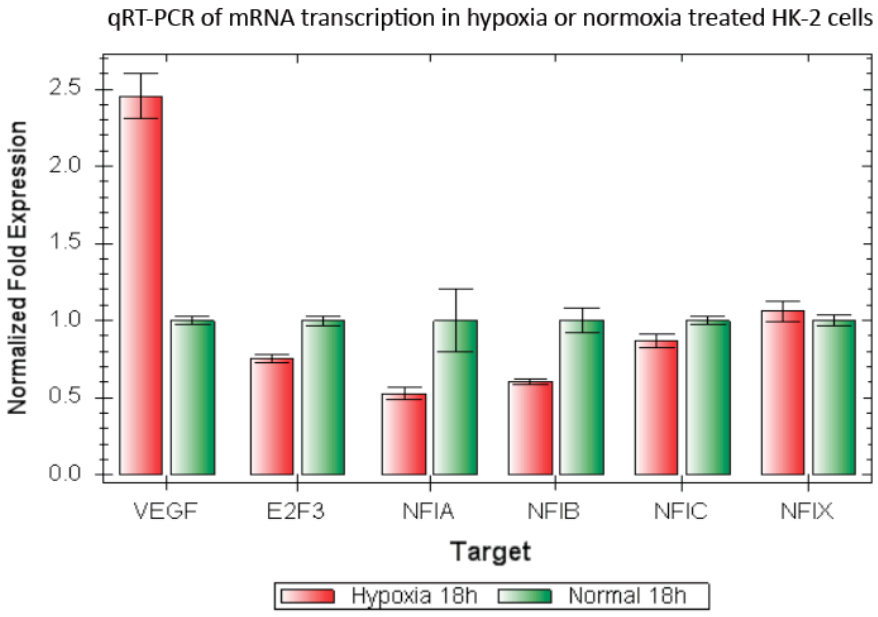
absence of an immune system and surrounding supportive cells (parenchymal cells) may alter the stress response from ischemia/reperfusion injury. An animal model is required to appropriately test the role of hypoxia-reperfusion on BKV reactivation.

Why does hypoxia inhibit BKV DNA replication, while re-oxygenation stimulates replication? First of all, the inhibition of BKV DNA replication by hypoxia is consistent with the reports that hypoxia decreases cell proliferation(29) and reduces the expression of cellular factors involved in DNA replication, such as PCNA, Cyclin D, E2F3, E2F6 etc.(14). Secondly, hypoxia dramatically changes overall gene expression of kidney tubular epithelial cells, through HIF1α-dependent and HIF1α-independent pathways(14, 17, 29). Pre-exposure of kidney cells to ischemia makes the cells less susceptible to subsequent ischemic insult, a process termed “ischemia pre-conditioning” (7). Studies of animal models indicate that the “ischemia pre-conditioning” is not only established through the change of differentiation states of kidney cells, but also through the long-term alteration of protein expression/activity in the affected cells (7, 35, 36), which may last for weeks(35). So, it possible that in spite of the inhibition of BKV DNA replication, hypoxia sustainably changes the expression or activity of some cellular factors which stimulates BKV DNA replication after re-oxygenation. Another possible but not mutually exclusive mechanism is that re-oxygenation following hypoxia activates the stress-response involving DNA damage/repair pathways, differentiation/regeneration of injured kidney tubules, which stimulates BKV DNA replication.

The following sections summarize and speculate how transcription factor NFI, histone acetyltransferases (HATs) and noncoding RNAs might modulate BKV DNA replication during ischemia/reperfusion in kidney allograft; future directions related to the hypothesis are proposed.

Figure 5.2 Expression of NFI, VEGF and E2F3 in HK-2 cells under normoxia (Green bars) and hypoxia (Red bars) conditions. The total RNAs in HK-2 cells treated under two conditions were extracted. The relative amount of mRNAs for each gene is determined by quantitative real-time RT-PCR (qRT-PCR); the gene expression under normoxia condition was arbitrarily set as 1.0; the expressions of all genes are normalized with expression of β-actin and 18S rRNA. Error bars are obtained from the standard deviation of three independent repeats.

Figure 5.2



NFI may regulate BKV DNA replication during ischemia/reperfusion.

In chapter 2, we have shown that NFI stimulates BKV DNA replication in HK-2 cells in competitive DNA replication assays; a specific NFI isotype, NFIC/CTF1 has been shown to stimulate BKV DNA replication *in vitro* through recruiting pol-α primase to origin of replication. NFI expression is ubiquitous and the expression of different isotypes is tissue specific and changes during development (11, 21, 28, 40). NFI can be either stimulatory or inhibitory depending on specific isotypes (12, 43), cell types and interaction with other factors. We have observed that isotype NFIC stimulates BKV DNA replication through recruitment of pol-α primase, but the effect of other NFI isotypes is unknown. Since NFIA interacts with BKV Tag, it is possible that NFI can also regulate BKV DNA replication through this interaction. We have shown that NFIA interacts with helicase domain of Tag; as p53 strongly inhibits SV40 DNA replication through interaction with Tag helicase domain and interference of helicase activity (30, 46, 48), it is possible that NFIA inhibits BKV DNA replication by a similar mechanism as p53. We have observed that hypoxia down-regulates transcription of NFIA and NFIB mRNA in HK-2 cells (Figure 4.2). Hypoxia may down-regulate the inhibitory NFIA or NFIB to stimulate BKV replication. This can be tested with *in vitro* BKV DNA replication assays by adding purified NFIA/NFIB and see if introduction of NFIA/NFIB inhibits the replication.

Chromatin remodeling activity of NFIC is modulated by TGF-β and TNF-α(1, 2, 27, 37); and DNA binding activity of NFI is controlled by oxidative stress (5, 31-33). TGF-β, TNF-α and oxidative stress are induced in kidney by ischemia/reperfusion injury (10, 18, 26). But how these are related to the stimulation of BKV DNA by hypoxia/re-oxygenation should be studied more carefully in the future.

HATs may regulate DNA replication during ischemia/reperfusion.

In chapter 3, we have shown that BKV/SV40 Tag can be acetylated and different HATs modulate BKV and SV40 DNA replication *in vivo* in different ways. Most remarkably, PCAF/GCN5 strongly inhibit BKV/SV40 DNA replication, in contrast to their stimulatory role on mPyV DNA replication. It has been recognized recently that HATs are extensively involved in the regulation of gene expression induced by inflammatory response to various stress related injuries, including ischemia/reperfusion insult; and many HDAC inhibitors are in clinical trails to examine their role in anti-inflammation (22). But how the activity and expression of HATs and histone deacetylases (HDACs) are modulated in ischemia/reperfusion has not been studied in detail. One report indicated that PCAF is down-regulated in brain ischemia/reperfusion injury(4); another study observed that HDAC1 is up-regulated in mice kidneys after hypoxia/reperfusion injury(17); and HDAC1 can deacetylate SV40 Tag, counteracting with PCAF (45). We postulate that ischemia/reperfusion may stimulate BKV DNA replication by down-regulating the inhibitory PCAF and up-regulating stimulatory HDAC1.

The function of BKV/SV40 Tags acetylation remains unknown (8, 38, 39, 45) (Chapter 2). Although the acetylation site is located within the host range (HR) domain, its does not seem to be related with the host range function of Tag (38). Up till now, all previous studies of Tag acetylation were carried out in cell culture model, which has limitations for study viral pathogenesis *in vivo*. We speculate that acetylation of Tag might be involved in BKV/SV40 reactivation *in vivo* during ischemia/reperfusion.

Noncoding RNAs may regulate BKV DNA replication during ischemia/reperfusion.

Some cellular noncoding RNAs were found to regulate BKV DNA replication (Chapter 4). Most interestingly, srRNAs act through BKV NCCR; and small RNAs in human cells stimulate BKV DNA replication. Dr. Irina Tikhanovich's preliminary data shows that srRNAs are naturally expressed in human and murine cells; and the sequences of these srRNA are similar to some small noncoding RNAs(snRNAs) expressed from heterochromatic and pericentromeric regions of

chromosome, which are greatly over-expressed in cancer cells and cells under stress or differentiation (9, 47). It is quite possible that ischemia/reperfusion injury may also induce the over-expression of these noncoding RNAs in kidney epithelial cells and stimulate BKV DNA replication. This hypothesis can be tested by deep sequencing of small RNAs induced in kidney cells by hypoxia.

Conclusion

Our preliminary results of hypoxia/re-oxygenation stimulating BKV DNA replication (Figure 5.1) suggests a model that ischemia/reperfusion injury can stimulate BKV DNA replication in kidney tubular epithelial cells, which may relate the pathogenesis of PVAN to three distinct regulatory mechanisms for BKV DNA replication as described in this dissertation (Chapter 2-4) and several published works (Mahon and Liang, *et al*., 2009; Tikhanovich *et al*., 2010; Tikhanovich and Liang, *et al*., 2011).

Future study should focus on how the hypoxia/re-oxygenation regulates BKV DNA replication through modulation of cellular transcription factors and/or noncoding RNAs.

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