Stimulation of BKV DNA replication by NFI-family transcription factors

Bo Liang¹#, Irina Tikhanovich²#, Heinz Peter Nasheuer², William R. Folk¹*

1. Department of Biochemistry and Genetics Area Program, University of Missouri-Columbia, Columbia, Missouri, 65211 USA;
2. Centre for Chromosome Biology, School of Natural Sciences, National University of Ireland, Galway, Galway, Ireland;
#current address: Laboratory of Infectious Disease, National Institute of Allergy and Infectious Diseases, National Institute of Health, Bethesda, Maryland, USA;
^current address: Department of Internal Medicine, University of Kansas Medical Center, 3901 Rainbow Blvd., Rm 3020, MS 3041, Kansas City, KS 66160

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* Corresponding author: Dr. William R. Folk, Department of Biochemistry, University of Missouri-Columbia, Columbia, Missouri, USA; Phone: 573-884-2921; FAX: 573-884-4597; email: folkw@missouri.edu
ABSTRACT

BK polyomavirus (BKV) establishes persistent, low level and asymptomatic infections in most humans but can cause polyomavirus associated nephropathy (PVAN) and other pathologies in some individuals. Activation of BKV replication following kidney transplantation, leading to viruria, viremia, and ultimately PVAN is thought to be due to immune suppression and to inflammation and stress from ischemia-reperfusion injury of the allograft; but the molecular mechanisms are not well defined. Replication of viral DNA in cell cultures is regulated by the viral noncoding control region (NCCR) comprising the core origin and flanking sequences, to which bind BKV T antigen (Tag), cellular proteins and small regulatory RNAs (45, 83). Six Nuclear Factor I (NFI) binding sites occur in sequences flanking the late side of the origin (the viral enhancer), and their mutation, either individually or in toto, reduced BKV DNA replication when placed in competition with templates containing intact BKV NCCRs. NFI family members interact with BKV Tag’s helicase domain in pull-down assays, suggesting NFI helps recruit Tag to the core origin and modulates its function. However, Tag may not be the sole target for NFI’s replication-modulatory activities: isotype NFI-C/CTF1 stimulation of BKV template replication in vitro is dependent upon the concentration of DNA polymerase-α primase (Pol-primase), and the p58 subunit of Pol-primase associates with NFI-C/CTF1, suggesting NFI recruits Pol-primase to the NCCR. We suggest that NFI proteins (and the signaling pathways that target them) help activate BKV replication.
INTRODUCTION

Human polyomavirus BK (BKV) persistently and asymptotically infects 80–90% of humans (24, 39). Kidneys are the major sites of replication, where BKV DNA is maintained at low levels (< 0.01 copy/cell on average) (19, 34). Activation of BKV replication in kidney allografts following transplantation increases viral titers to >1000 copies/cell (73) with concomitant viruria, viremia and occasionally, polyomavirus associated nephropathy (PVAN), a major source of allograft loss. BKV persistent infections require down-regulation of the viral T-antigen and the cellular stress-induced ligand ULBP3, (Seo et al, 2008, J. Virology 82:9823; Bauman et al, 2011, Cell Host and Microbe 9: 93-102) but the causes and mechanisms for activation of viral replication are not understood.

BKV replication is controlled by the viral noncoding control region (NCCR), within which the “core origin” (core-ori) serves as the initial binding site for the viral initiator-helicase protein, T antigen (Tag)(20, 69) (Fig 1). Adjacent to the core-ori are the early flanking (EF) and the late flanking sequences (the “enhancer”) to which histones and cellular transcription factors and small noncoding RNAs (Figure 1) bind and that control viral gene transcription and DNA replication (45, 51, 83, 84). The BKV archetype enhancer, comprising four single-copy sequence blocks, termed P_{68}, Q_{39}, R_{63}, S_{63} (Figure 1), occurs in strains WW and Dik, but rearranges by duplication, deletion and insertion in late stage PVAN or after passage in cell culture, providing replication advantage or perhaps enhanced tropism (9, 29, 76).

Binding sites for numerous cellular transcription factors, including NFI (13-15, 21, 46), Sp1 (13, 21, 46), NFAT (38), AP1 (14, 21, 46), Smad3 (1), ERE and GRE/PRE
(52), p53 (79), NFκB (27) and C/EBP (27) have been identified in the archetype BKV enhancer (Figure 1) and variants, and evidence supports the importance of some of these for viral transcription and replication. Also, putative binding sites for Ets-1, PEA3, AP-2, CREB, and GM-CSF have been predicted by sequence homology (51, 74), but their functional importance is uncertain. Notably, multiple NFI binding sites occur in the BKV archetype (Figure 1) as well as in rearranged enhancers (13, 21, 46), suggesting they may be functionally important. While some NFI sites have been shown to regulate BKV early/late promoter activity (14, 15, 30, 40), the involvement of NFI sites in viral DNA replication has not been demonstrated.

NFI was originally identified as a cellular factor that stimulates adenovirus DNA replication by recruiting the viral DNA polymerase to the viral origin of replication and distorting its structure (18, 62, 64). Subsequent studies indicated NFI is comprised of four isotypes, NFI-A, NFI-B, NFI-C and NFI-X (or NFI-D) with almost identical N-terminal DNA binding/dimerization domains that bind to “TGGN_{5-7} GCCAA” sequences (31, 32). The expression pattern of NFI isotypes is cell-type dependent and changes during differentiation and development (16, 41). NFI binding sites occur in many cellular promoters and enhancers as well as in viral genomes including BKV (13-15, 21, 46), human JC virus (JCV) (55), variant murine polyomavirus (mPyV) (12, 85) , HPV (68), HSV-1 (42), and CMV (33). The functional importance of these NFI sites in regulation of gene transcription is well established, but whether they also regulate DNA replication (other than adenoviral) has not been demonstrated.
Here, we provide evidence for the functional importance of NFI in the BKV archetype DNA replication: NFI sites proximal to the core-ori stimulate BKV DNA replication and mutation of NFI sites in the BKV enhancer diminishes BKV DNA replication in competitive DNA replication assays. Furthermore, NFI interacts with BKV Tag and the p58 subunit of DNA polymerase-α primase (Pol-primase), as detected by \textit{in vitro} pull-down assays and co-immunoprecipitation (co-IP) assays respectively, and NFI-C/CTF-1 stimulates BKV DNA replication \textit{in vitro} when Pol-primase is limiting.
MATERIALS AND METHODS

Plasmids. Test replication templates pUC-wt-BKV and pUC-Δen-BKV were generated by insertion of PCR fragments of the intact NCCR (positions 5031 to 282) and NCCR without enhancer (positions 5031 to 32) of archetype BKV (Dik strain; GenBank Accession #AB211369) into the XmaI/PstI sites of pUC18. pUC-6mtNFIs-BKV was generated by ligation of synthesized mutant BKV NCCR (GenScript) into the XmaI/PstI sites of pUC18. pUC-5mtNFIsW1-BKV, pUC-5mtNFIsW2-BKV, pUC-5mtNFIsW3-BKV and pUC-5mtNFIsW6-BKV were derived from pUC-6mtNFIs-BKV using the QuickChange Site-Directed Mutagenesis kit (Stratagene). To construct test templates pUC-BKV-1fNFI, pUC-BKV-1rNFI, pUC-BKV-2rNFI, pUC-BKV-4rNFI, synthetic oligonucleotides 5-CACATGGGAATGTAGCCAAAACGTGCA-3 and 5-GTTTGGCTACATTCCATGTGTGCA-3 were annealed and self-ligated and inserted into the PstI site of pUC-Δen-BKV. Competition templates pBC-wt-BKV, pBC-BKV-en and pBC-BKV-ori were generated by insertion of PCR fragments of the intact NCCR (positions 5031 to 282), enhancer (position 33-282) and core origin (positions 5103 to 32) of BKV (Dik) into the XmaI/PstI, NotI/PstI and NotI/Xhol sites of pBC-Sk(+), respectively. pBC-BKV-A89G, pBC-BKV-A143G, pBC-BKV-A141T were mutated using the QuickChange Site-Directed Mutagenesis kit.

The mammalian expression vector for BKV Tag, pCMV-BKT-Flag, lacking an SV40 origin has been described (45). NFI expression vectors pCH-NFIA, pCH-NFIB, pCH-NFIC, pCH-NFIX and pCH-empty were kindly provided by Richard Gronostajski (17). pCH-hNFIC/CTF1 was generated by insertion of the human NFIC/CTF-1 cDNA (amplified by PCR from pCMV-CTF-1ΔUTR (kindly provided by Nicolas
Mermod) (48) using primers 5'-AGCTGGGCCCATGGATGAGTTCCAC-3' and 5'-TTGCGCTAGCCTATCCCAAGATACCAGGAC-3') into NheI/ApaI sites of pCH-empty vector. The Pol-primase subunits were cloned into the expression vector pCMV with a T7 epitope tag at their N-termini. The expression plasmids were kindly provided by Drs. Sock and Wegner (University of Erlangen-Nürnberg).

Bacterial expression vectors for truncated BKV Tag (pGEX3X-BKTHD, pGEX3X-BKTHDHR, pGEX3X-BKTHR) were generated by insertion of PCR amplified fragments into EcoRI/BamHI sites of the pGEX3X vector. Primers used for PCR were: BKTHD 5'-ATAGGATCCCCAGCTAAAGGAGCATGATTTTAAC-3' and 5'-CGGCCAATTCTTAAATCAAGAATACATTTCCTTTCATG-3'; BKTHDHR 5'-ATAGGATCCCCAGCTAAAGGAGCATGATTTTAAC-3' and ACGCGAATTCTTATTTGGGGTGGTGTTTTAG-3'; BKTHR 5'-ATATGGATCCCCACATTACAGAAGAGGATTCAG-3' and 5'-ACGCGAATTCTTATTTGGGGTGGTGTTTTAG-3'.

**Cell cultures and antibodies**

HK-2 human proximal tubular epithelial cells were cultured in RPMI medium 1640 (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone), 4 mM L-glutamine and 100 µg/ml penicillin and 100 µg/ml streptomycin (Lonza). HEK293 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 100 µg/ml penicillin and 100 µg/ml streptomycin (Lonza). RPTECs (renal primary tubular epithelial cells) were cultured in Renal Epithelial
Growth Medium with supplements (Lonza). All cells were grown at 37°C with 5% CO₂ in a humidified incubator.

Antibodies used in Western Blots: anti-NFI (Santa Cruz, sc-870), anti-p53 (Cell Signaling), anti-c-Jun (Santa Cruz, sc-1694), anti-Sp1 (Santa Cruz, sc-59), anti-Ets1 (Santa Cruz, sc-111), anti-NFκB (Santa Cruz, sc-372), anti-CREB (Santa Cruz, sc-58), anti-Smad3 (Santa Cruz, sc-8332), M2 anti-Flag (Sigma), anti-β-actin (Santa Cruz, sc-47778), anti-HA (Roche), anti-T7 (Novagen). Polyclonal rabbit antiserum recognizing p58 and p48 was produced by injecting recombinant human primase p58-p48 expressed in *E. coli* into rabbits and purified as previously described. (Weisshart et al., 2000)

**DNA replication assays**

DNA replication assays in transfected cells were performed as previously described (45, 83), with transfection procedures optimized by luciferase/β-Galactosidase reporter assays. The *in vitro* monoplymerase assays were performed as previously described (45, 84) with slight modifications: the assay included 0.5 μg of BKV template DNA, 50 ng topoisomerase I, 1 μg RPA, and Pol-primase (as indicated in the figure legend), in 30 mM HEPES 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 10 μM 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 amount of NFI protein is indicated in the figure legend. Purified BKV Tag (0.4 μg) was added to start the reaction 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.

**Nuclear extracts**

HEK293 cells from 10 liters of suspension culture were purchased from The National Cell Culture Center (NCCC), Minneapolis, USA. Cell pellet was washed in 5 packed cell volume (pcv) of ice-cold hypotonic buffer (10 mM HEPES, pH7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.2 mM PMSF, 0.5 mM dithiothreitol (DTT)) and centrifuged in a Beckman GH-3.7 rotor at 3000 rpm for 5 minutes at 4°C; pelleted cells were resuspended in 3 pcv of ice-cold hypotonic buffer and allowed to swell on ice for 10 minutes; swollen cells were transferred to a chilled glass Dounce tissue grinder and homogenized with 10-15 strokes using type B pestle and centrifuged in a Beckman GH-3.7 rotor at 3500 rpm for 15 min in 4°C; the supernatants were removed and the pellets were resuspended in 1 packed nuclear volume (pnv) of low salt buffer (20 mM HEPES, pH7.9, 25% glycerol, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT); 0.183 pnv of 5 M NaCl was added dropwise to the resuspended nuclei while mixing gently by swirling and placed on a rotating platform for 30 min at 4°C and centrifuged at 16000 rpm (37,000×g) in a Sorvall SA-600 rotor for 1 h at 4°C. The supernatants were collected and dialyzed in dialysis buffer (20 mM HEPES, pH7.9, 20% glycerol, 5 mM NaCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT). The protein concentration of extracts was 1.4 µg/µl 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).

**Competitive electrophoretic mobility shift assays (EMSAs)**

Competitive EMSAs were performed following published procedures (10) with a few modifications: 4 pmol of competitor oligonucleotides, 2 µg of HeLa nuclear extracts (sc-2120, Santa Cruz) or 0.25 µg of purified hNFIC/CTF-1 (Abcam), 50 ng of poly(dI.dC) were incubated in 1X HEPES Binding Buffer (25mM HEPES, pH7.5, 6 mM MgCl₂, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 0.5 mM PMSF, 5% Glycerol) for 10 minutes at room-temperature (R.T.); then, 20 fmol of biotin-labeled probe for the NFI site was added to the reaction and incubated for 20 min at R.T. (200-fold excess based upon empirical values reported by others (40, 77)). For antibody super-shift assays, 1 µl of NFI antibody (sc-5567, Santa Cruz) was added after the binding reaction and incubated for another 10 min at R.T. The reaction products were fractionated in 5% non-denaturing polyacrylamide gels (pre-run in 0.5XTBE at 100V for 30 min) in 0.5XTBE at 100V until the bromophenol blue dye reached the bottom of the gel. The products were transferred to nylon membranes in 0.5XTBE at 65 V for 30 min and UV cross-linked for 15 min and visualized by chemiluminescent nucleic acid detection (Pierce).

**In vitro pull-down assays**

*Full-length Tag pull-down assays:* BKV Tag with N-terminal Flag epitope was expressed using the Bac-to-Bac Baculovirus System (Invitrogen). 1.5X10⁷ infected Hi-Five insect cells were harvested 48 h post-infection (p.i.) and lysed in 1ml of 0.5%
NP-40 lysis Buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 5 mM KCl, 1.0 mM MgCl₂, 0.5% NP-40, 10% glycerol, 1X PhosSTOP phosphatase inhibitors (Roche), 1X complete protease inhibitor cocktail (Roche)) by incubating on a rotating platform at 4°C for 30 minutes and homogenization with glass Dounce tissue grinder. Lysates were cleared by centrifugation at 20,000 × g in a Sorvall SA-600 rotor for 30 minutes in 4°C. Supernatants were incubated with 60 µl of anti-Flag M2 Affinity Gel (Sigma) by rotating at 4°C for 2 h and washed three times with 1 ml of ice-cold PBS; then, each of the gel suspensions incubated with extracts of infected and uninfected Hi-Five cells was incubated with 1 ml of HEK293 nuclear extracts (1.4 µg/µl) plus 1X complete protease inhibitor cocktail (Roche) by rotating in 4°C for 12 h. The gel was washed three times with 1 ml of ice-cold PBS and boiled 5 min with 50 µl of 1X SDS sample buffer.

**Truncated Tag pull-down assays:** GST-BKV Tag proteins were expressed in Rosetta™ 2 E.coli cells (Novagen) cultured in LB medium on a shaking platform at 225 rpm at 25°C. Expression was induced with 1 mM IPTG when A₆₀₀ reached 0.4-0.5. After induction, E. coli were cultured at 25°C with 225 rpm shaking for 20 h, and then sonicated (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 DTT, 10% glycerol, 1 mM PMSF, 1X complete protease inhibitor cocktail (Roche)). 0.1% NP-40 was added to the lysates after sonication and incubated on ice for 10 min. After centrifugation at 20,000 × g in Sorvall SA-600 rotor for 30 minutes at 4°C, supernatants were incubated with glutathione beads (GE Health) at 4°C for 2 h followed by washing 2 times with 20 bed volume of buffer L1. A small portion (1/20) of the beads for each fusion protein was boiled with SDS sample buffer to check the quantity of bound fusion
proteins by SDS-PAGE and Coomassie Blue staining. Beads with approximately equal amount of each fusion protein (~50 µg) were incubated with 800 µl of HEK293 nuclear extract (1.4 µg/µl) supplemented with 1x complete protease inhibitor cocktail (Roche) by rotating at 4°C for 12 h. The beads were washed 5 times with 1ml of buffer L1, and then boiled 5 minutes with 100 µl 1X SDS sample buffer. All samples were fractionated by SDS-PAGE and analyzed by Coomassie Blue staining, followed by western blotting.

**Co-immunoprecipitation assays**

HEK293 cells (approximately 6x10^6) were transfected with expression vectors by using LipofectAMINE and PLUS reagent (Invitrogen) in 100 mm-diameter plates as previously described (45). At 48 h P.T, the cells were lysed with 750 µl of 1% Triton lysis buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 30 µM ethidium bromide, 1 mM 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 20,000×g in a Sorvall SA-600 rotor for 30 minutes at 4°C. A sample (10 µl) was taken for protein input control, and the remaining supernatants were incubated with 50 µl of Anti-Flag M2 Affinity Gel (Sigma) or 50 µl of Anti-HA Affinity Matrix (Roche) by rotating at 4°C for 2 h, followed by four washes with 1ml of 1% Triton lysis buffer. Following the last wash, pelleted beads were suspended in 40 µl of 1X SDS sample buffer, boiled for 5 minutes. Samples of 20 µl of the supernatant were loaded for SDS-PAGE and western blotting.
RESULTS

Synthetic NFI sites proximal to the core origin stimulate BKV DNA replication in HK2 cells

Six putative NFI binding sites have been identified in the BKV archetype enhancer (Dik strain), which are named from NFI-1 to -6 according to their distance from the core origin (Figure 1). To determine if NFI proteins modulate BKV Tag-dependent DNA replication, synthetic NFI-1 sites corresponding to the first NFI site in the P block (Figure 1) in different orientations and copy numbers were substituted for enhancer sequences (Figure 2A) and assayed for Tag-dependent DNA replication in HK2 cells (Figure 2B lane 2-5). The addition of NFI sites to templates lacking the enhancer stimulated replication, with the extent of activation correlating with the number of sites (Figure 2B lane 6-8), but not their orientation (Figure 2B lanes 3 and 4). Replication of templates containing multiple NFI sites was always weaker than replication of templates with the archetype enhancer (Figure 2B, lanes 1, 5, 7, and 8), suggesting other elements also stimulate BKV DNA replication in these cells or that a particular spatial configuration of NFI sites is required.

Mutation of the BKV enhancer NFI sites reduces NFI binding

A competitive EMSA was employed to evaluate NFI binding to the NFI sites in the BKV enhancer. As a positive control for NFI protein-oligonucleotide complexes, EMSA was performed with a biotin-labeled oligonucleotide with the sequence of the NFI site in the adenovirus ITR (inverted terminal repeat) (43); as expected, a shift of the migration of the oligonucleotide caused by NFI binding was observed in the absence of competitor (Figure 3A, lanes 1 and 6), and addition of 200x excess of
unlabeled oligonucleotide fully competed NFI binding (Figure 3A, lanes 2 and 7); however, an oligonucleotide with a point mutation in the NFI site failed to compete NFI binding and did not alter the band shift (Figure 3A, lane 3). Adding anti-NFI antibody to the binding reaction caused a super shift (dashed-arrow in Figure 3A, lane 4), confirming the band shift was caused by NFI. An excess of BKV enhancer DNA also competed for NFI binding (Figure 3A, lane 5), confirming that NFI binds to the BKV archetype enhancer. Oligonucleotides containing sequences of the six NFI binding sites in the BKV enhancer (Figure 3B) were able to compete for NFI binding, but with different efficiencies (Figure 3A, lane 8-13), suggesting these sites have different affinities for NFI proteins. Sequences of NFI sites closer to the core-ori competed more efficiently than distal sites.

Point mutations were introduced into the NFI consensus sequence “TGGN_{5-7}GCCAA” of each of the six NFI sites in the enhancer, and the design of the mutations was confirmed with MatInspector (72) to ensure that no new transcription factor binding sites were created (Figure 3B). Oligonucleotides with mutant sequences were demonstrably defective for NFI binding in competitive EMSAs (Figure 3C). A 200 fold excess of the BKV enhancer DNA with mutations in all six NFI sites (6mtNFIs BK enhancer) only slightly reduced the NFI binding to the adenovirus ITR oligonucleotide (Figure 3D, compare lanes 1 and 3) whereas the wild type enhancer sequence completely abolished NFI binding (Figure 3D, lane 2). Some additional weak competition by the 6mtNFIs BK enhancer sequence was observed with 400 and 600-fold molar excess of oligonucleotide (Figure 3D, lanes 4 and 5). Moreover, the 6mtNFIs BK enhancer sequence had little effect on binding of adenovirus ITR by purified NFI-C/CTF1 using a 200-fold molar excess, whereas the wild type BKV
enhancer effectively competed binding (Figure 3E). These results indicate that mutation of the six identified NFI sites greatly reduces NFI binding to the BKV enhancer; however, the mutant BKV enhancer may contain additional cryptic NFI binding site(s), or alternatively, NFI may bind to the mutant enhancer cooperatively with other transcription factors in the nuclear extracts.

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

Assays of the replication of BKV templates with mutations of all six NFI sites (pUC-6mtNFIs-BKV) compared with replication of wild type template (pUC-wt-BKV) in HK-2 cells transfected with each template separately, indicated both templates replicated with similar efficiency (data not shown). However, in a competitive assay, in which a competitor (3.4kb) template pBC-wt-BKV with the archetype NCCR was co-transfected into HK-2 cells together with test templates (2.6 kb) (Figure 4A) and a Tag expression vector (pCMV-BKT-Flag), replication of the test template with the archetype enhancer containing six mutant NFI sites (pUC-6mtNFIs-BKV) was greatly reduced (Figure 4B, compare lane 3 with lane 1), as also was observed for a test template without enhancer (Figure 4B, lane 2). Test templates with mutations in Ets-1 binding sites in the enhancer (pUC-mtEts1s-BKV) replicated with similar efficiency as the wild type template (Figure 4B, compare lane 5 with lane 1), indicating specificity in the effect of the NFI site mutations upon DNA replication in the competitive assay.

To determine whether a specific NFI site(s) of the six identified in the archetype enhancer is responsible for the stimulatory effect upon DNA replication in the competitive assay, templates with several mutated NFI sites reverted to wild type
were assayed for replication; the results suggested that NFI sites closer to core-ori are functionally more important for BKV DNA replication (Figure 4C, lanes 3-7).

The requirement of a competitor template to observe the stimulatory effect of NFI sites for BKV DNA replication might be explained by NFI recruiting factors required for DNA replication, that are made limiting by use of the competitor template. The observation that NFI sites closer to core-ori are more important for replication than distal sites suggests that NFI may target components of the initiation complex, such as Tag, Pol-primase, replication protein A (RPA) or Topoisomerase I. Evidence favoring these notions is provided by the experiments described below.

**NFI interacts with BKV Tag**

We tested whether NFI interacts with BKV Tag by antibody mediated pull-down assays. Full-length Flag-tagged BKV Tag (BKT-Flag) was mixed with HEK293 nuclear extracts and protein complexes were collected using Flag antibody resin and associated proteins detected with antibodies (Figure 5A). NFI, p53, Sp1 and c-Jun, but not Ets1, CREB, NFKB p65 or Smad3 associated with BKV Tag (Figure 5A). Specific domains of BKV Tag that interact with these transcription factors were determined with GST-tagged truncated BKV Tags (Figure 5B). The BKV Tag helicase/ATPase domain (HD) pulled down NFI, p53, c-Jun strongly and Sp1 weakly, but not CREB, NFKB (Figure 5C, lane 4 and 5), indicating that NFI, p53 and c-Jun interact with the helicase/ATPase domain, while Sp1 may also interact with other Tag domains in addition to HD. These observations are consistent with reports that BKV Tag complexes with p53 (79) and that the highly homologous SV40 Tag HD interacts with p53 (44), the SV40 Tag OBD interacts with Sp1 (37) and SV40 interacts with c-
None of the transcription factors tested appeared to interact with the BKV Tag C-terminal region (Figure 5C lane 3). Truncated BKV Tags did not pull down factors not observed to complex with full-length BKV Tag, including Ets1, NFkB, CREB or Smad3 (Figure 5A, C; data not shown).

To determine whether other isotypes of NFI also interact with BKV Tag, HA-tagged NFI isotypes and BKV Tag were over-expressed in HEK293 cells. The interaction of Tag with all four NFI isotypes was detected with Co-IP assays immunoprecipitated by either anti-HA or anti-Flag antibodies (data not shown).

**Increasing Tag expression only partially rescue the replication deficiency of BKV template with mutant NFI sites**

The interaction of BKV Tag with NFI provides a possible basis for the stimulatory effect upon replication of NFI sites in the enhancer. Initially, BKV Tag was expressed at low level in the competitive DNA replication assays (Figure 4B); to investigate if BKV Tag is the only limiting factor in these assays, increasing amounts of pCMV-BKV Tag expression vector were introduced into HK-2 cells and DNA replication of wild type and mutant (NFI site) templates compared (Figure 6). BKV Tag expressed from 400ng of pCMV-BKTag saturated replication (Figure 6 lane 7-9), but this amount and even addition of 900ng pCMV-BKTag only partially rescued the replication of the template with the mutant NFI sites (pUC-6mtNFI-BKV) compared with robust replication of wild type template (pUC-wt-BKV) (Figure 6, compare lane 9 with 7, and compare lane 12 with 10). Furthermore, the template with a deleted enhancer (pUC-Δen-BKV) replicated poorly when BKV Tag was highly expressed.
(Figure 6, lanes 8 and 11). These observations indicate that high levels of BKV Tag alone cannot correct the replication deficiency of templates with mutant NFI sites, and suggest that BKV Tag is not the sole limiting factor in these replication assays.

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

BKV Tag interacts with all different NFI isotypes in Co-IP assays (data not shown), but the function of the interaction for BKV replication is difficult to test *in vivo* due to the expression of endogenous NFI isotypes, as we analyzed their expression in HK-2, HeLa, HEK293 and primary kidney tubular epithelial cells (data not shown). Isotype NFI-C/CTF1 stimulates initiation of adenovirus DNA replication by recruiting adenovirus DNA polymerase to the origin of replication (8, 18); and the proline-rich transactivation domain of isotype NFI-C/CTF1 stimulates SV40 DNA replication when tethered to the SV40 origin (61); and NFI-C is expressed in HK-2 cells at similar level as in primary kidney epithelial cells (data not shown). These observations prompted us to assess whether NFI-C/CTF1 can stimulate BKV DNA replication in the monopolymerase system that contains Pol-primase, RPA and Topoisomerase I (45, 84) in the absence of other cellular factors.

In support of NFI targeting a component of the monopolymerase system, when Pol-primase was limiting *in vitro* the DNA replication of wild type BKV template was stimulated strongly by NFI-C/CTF1 in a dose-dependent manner (Figure 7, lanes 4-6). In contrast, with high levels of Pol-primase, adding NFIC/CTF1 had no effect upon initiation of wild type BKV template (Figure 7, lanes 1-3). Furthermore, no stimulation was observed with the NFI binding site-mutant BKV template regardless
of Pol-primase levels (Figure 7, lane 7-12). These findings indicate that NFI-C/CTF1 stimulates initiation of BKV DNA replication \textit{in vitro} only when Pol-primase is limiting, and suggests Pol-primase is a limiting factor targeted by NFI to stimulate BKV DNA replication \textit{in vivo} in the competitive replication assays.

**NFI-C/CTF1 interacts with the p58 subunit of cellular DNA polymerase-α primase**

To investigate the mechanism of this functional NFI-Pol-primase interaction we studied whether these proteins form a complex in human cells. Pol-primase consists of four subunits: two smaller subunits, p48 and p58 constitute the primase; and two larger subunits, p180 and p68 constitute the DNA polymerase-α catalytic plus the regulatory subunit p68 (49, 50, 66, 81). HA-tagged human NFI-C/CTF1 was over-expressed with each of four T7-tagged human Pol-primase subunits in HEK293 cells (Figure 8A); the interaction of NFI-C/CTF1 with these subunits was examined with co-IP assays. Expression of p180 and p68 subunit was much more efficient than expression of each of the two primase subunits (as detected with anti-T7 antibody; Figure 8A, lane 3-6), but no interaction was detected between NFI-C/CTF1 and either p180 or p68 (data not shown). Because p58 expression was low (Figure 8A, lane 4), a more sensitive antibody against p58 was used instead of the anti-T7 antibody for detection of p58 (Figure 8B). In this assay p58 was found to co-precipitate with NFI-C/CTF1 (Figure 8B, lane 1, panel WB: anti-p58), and p58 co-IP was not detected in three control reactions, in which p58 (Figure 8B, lane 2, panel WB: anti-p58) or NFI-C/CTF1 (Figure 8B, lane 3, panel WB: anti-p58) or neither (Figure 10, lane 4, panel WB: anti-p58) was expressed. No other significantly distinct band was detected in the co-precipitated fraction. The expression of p48 was extremely low and a physical
interaction between p48 and NFI-C/CTF1 was not detected (Figure 8A, lane 3; data not shown), but we cannot exclude that a small amount of p48 might bind to NFI.

DISCUSSION

After almost two decades since the first report of PVAN and identification of its etiology as being due to activation of BKV replication, the responsible mechanism(s) for activation of BKV replication in kidney allografts still remain elusive. Immune suppression appears to be partially responsible for activation of BKV (2, 25, 87), and involvement of cellular factors induced by stress related injury, repair, regeneration and differentiation also are likely (26, 35). Understanding how these processes act upon the viral NCCR to regulate BKV replication should provide insights and assist development of strategies to mitigate PVAN and other pathologies cause by BKV.

We have identified six NFI sites in the BKV archetype NCCR by competitive EMSA (Figure 3A lane 8-13) and have found that two NFI sites close to core origin in P_{24-37} (NFI-1) and P_{68-Q_{13}} junction (NFI-2) have higher affinity to NFI (Figure 3A lane 8-13) and stronger stimulatory effect on BKV DNA replication than distal sites (Figure 4C, lane 4-7). Almost all rearranged viruses contain the P block and P-Q junction region spanning the first two NFI sites (29, 53, 70, 71, 74), suggesting these two NFI sites might be particularly important for efficient viral replication \textit{in vivo}. Other NFI sites have been implicated in the early-late transcription switch (NFI-3) (40), regulation of viral gene transcription in response to induction of TGF-β in kidney allografts (NFI-4) (1), and modulation of hormone-mediated stimulation of BKV replication (NFI-5 and -6) (52). We have attempted to distinguish the binding by different NFI isotypes using isotype-specific antibodies. Unfortunately, none of the
available isotype-specific antibodies work in EMSAs. As NFI isotypes have identical DNA binding domain that determines the binding specificity to consensus sites, we speculate that the different NFI isotypes have similar binding affinities in vitro. However, the actual binding activity of NFI isotypes in vivo is likely affected by their interaction/competition with other transcription factors that bind nearby in response to stimuli (6, 9, 11), and is challenging to demonstrate with in vitro assays using purified proteins.

The NFI sites in the archetype BKV enhancer resemble NFI sites in the archetype JCV enhancer, except that JCV lacks NFI-4 overlapping the Smad3 site (47). The NFI site closest to the core origin of JCV also stimulated JCV DNA replication in vivo (80). JCV also persistently infects kidney; and reactivation of JCV in immune-compromised individuals causes progressive multifocal leukoencephalopathy (PML) (86). Analysis of JCV rearranged enhancers in PML patients also revealed a trend similar to that with rearranged BKV enhancers in PVAN: sequences close to the core origin (A to C for JCV; P and P-Q junction for BKV), which contain the first two NFI sites (NFI-1 and NFI-2), are usually preserved and duplicated (28, 29). NFI isotype-specific expression determines the tropism of JCV (54, 75), but functions for different NFI isotypes have not been identified in vivo replication assays.

Characterization of NFI isotypes-specific function for BKV DNA replication has been attempted with in vivo replication system, but the results is complicated by the endogenous expression of different NFI isotypes/splicing variants (data not shown). Using the in vitro monopolymerase assay, we have defined the stimulatory activity of isotype NFI-C/CTF-1, the prototype of NFI-family transcription factors, for initiation
of BKV DNA replication (Figure 8). The role of other NFI isotypes in DNA replication will be tested in similar systems as purified NFI isotype proteins become available.

As NFI stimulates adenovirus (Ad2/5) DNA replication through recruitment of Ad pol-pTP complex (adenovirus DNA polymerase-preterminal protein) to the replication origin (8, 18, 60) and/or stabilization of pre-initiation complex (59), we suggest similar mechanisms promote BKV DNA replication through recruitment of BKV Tag and Pol-primase to the replication origin (Figure 10). In support of this, we observed that NFI stimulated initiation of BKV DNA replication \textit{in vitro} only at (low) equimolar concentration of Pol-primase and NFI, whereas at high concentrations of Pol-primase no stimulation was observed (Figure 8, lane 1-6). This is reminiscent of the stimulation of adenovirus DNA replication \textit{in vitro} by NFI which also depends on the concentration of pol-pTP (60). And this is also consistent with the results of \textit{in vivo} replication assays indicating the stimulatory effect of NFI sites is observed only in a competition assay (Figure 4B and C; data not shown). Furthermore, NFI forms complex with BKV Tag (Figure 5A and data not shown) and Pol-primase (Figure 8B). Swine NFI physically binds to calf primase and stimulates primase activity in a concentration-dependent manner in a biochemical assay (23), and all four NFI isotypes were found to interact with BKV Tag in co-IP assays (data not shown). These results suggest the formation of NFI-primase and NFI-Tag complexes might be important for initiation of DNA replication and their functions are complex due to co-expression of different NFI isotypes/splicing variants. Further studies are planned to characterize the nature and function of these interactions.
Although the NFI sites are not required for BKV DNA replication in the absence of a competitor template, NFI sites in the enhancer stimulate BKV DNA replication when Tag or Pol-primase is limiting (Figures 2B, 4B and 9). This stimulatory activity might be essential for reactivation of BKV replication as well as for persistent infection, where BKV replicates at very low levels in kidney tubular epithelial cells when low levels of Tag are expressed (19, 34, 73). The tubular epithelial cells in normal kidneys are terminally differentiated quiescent cells dividing at a low rate (7, 63), and express low amounts of Pol-primase (82). Ubiquitously expressed NFI may facilitate the low level replication of persistent BKV in kidney epithelial cells by increasing the apparent level of Pol-primase at the origin. Also, stress-related signaling mediated through TGF-β (3, 4), TNF-α (4), oxidative stress (5, 56-58) induced by kidney ischemia/reperfusion injury and/or inflammatory responses (11, 22, 36) during kidney transplantation or by administering of immunosuppressive drugs (such as Tacrolimus and Cyclosporine A) (67) might change the NFI isotype expression or their activity and thereby promote the NFI-mediated recruitment of Tag and/or Pol-primase to the viral core-ori and facilitate the assembly of pre-initiation complexes (Figure 10) or modulation of Tag’s and Pol-primase’s activity.

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Figure 1. BKV NCCR and transcription factor binding sites in the viral enhancer. Organization of BKV NCCR and sequence of the archetype BKV enhancer (Dik) and its transcription factor binding sites; the six NFI sites are highlighted and numbered from NFI-1 to NFI-6, with the first proximal to the BKV core origin.
Figure 2. DNA replication of BKV templates with synthetic NFI sites in place of the enhancer. (A) Design of BKV templates with synthetic NFI sites in place of the enhancer; (B) Southern blot of DNA replication assays in HK2 cells with BKV templates containing synthetic NFI sites: 10 ng BKV template and 10 ng pCMV-BKT-Flag were transfected into HK2 cells with carrier DNA; at 48 h p.t. cells were harvested and DpnI resistant, replicated DNA was analyzed by Southern blot.
Figure 3. Competitive EMSA assays. (A) Competitive EMSA using the NFI site in Ad ITR as probe. Lane 1, in the absence of competitor; lane 2, 200x molar excess of oligonucleotide with NFI site in Ad ITR as competitor; lane 3, 200x molar excess of oligonucleotide with mutant NFI site Ad ITR as competitor; lane 4, in the absence of a competitor, but incubated with anti-NFI antibody for 10 min after start of binding reaction; lane 5, 200x molar excess of PCR fragments of BKV enhancer as
competitor; lane 6, same as lane 1; lane 7, same as lane 2; lane 8-13, 200x molar excess of oligonucleotides of NFI sites in BKV enhancer as competitors. Positions of free probe and shifted probe are indicated with arrows. (B) Sequences of oligonucleotides for NFI sites used in EMSA. NFI binding motifs were underlined. Point mutations are indicated above the mutated positions; (C) Competitive EMSA with wild type (wt) NFI sites and mutant (mt) NFI sites in BKV enhancer. (D) Competitive EMSA with mutant BKV enhancer containing six mutated NFI sites (6mtNFIs). Lane 1, in the absence of competitor; lane 2, with wild type BKV enhancer PCR fragment as competitor; lane 3-5, with increasing amount of 6mtNFIs mutant BKV enhancer PCR fragment as competitor (200x, 400x and 600x molar excess of oligonucleotide which is equivalent to 4, 8 and 12 pmol, respectively). (E) Competitive EMSA using purified NFI-C/CTF1 instead of HeLa nuclear extracts. Lane 1, in the absence of competitor; lane 2, 200x molar excess of wild type BKV enhancer PCR fragment as competitor; lane 3, 200x molar excess of 6mtNFIs mutant BKV enhancer PCR fragment as competitor.
Figure 4. Competitive DNA replication assays in the presence of a wild type BKV template (pBC-wt-BKV). 25 ng pUC-template, 100 ng pBC-wt-BKV competitor and 25 ng pCMV-BKT-Flag were transfected into HK-2 cells with carrier DNA; 48 hour p.t. cells were harvested and DNA replication was analyzed by Southern blot. (A) Illustration of template and competitor for competitive DNA replication assays. (B) Lane 1, wild type 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 wild type (W6) (pUC-5mtNFIsW6-BKV); lane 5, Ets1 sites mutant BKV template (pUC-mtEts1s-BKV). (C) Lane 1, wild type 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 wild type (W6) (pUC-5mtNFIsW6-BKV); lane 5, Ets1 sites mutant BKV template (pUC-mtEts1s-BKV).
Δen-BKV); lane 3, 6mtNFIs mutant BKV template (pUC-6mtNFIs-BKV); lane 4, 5mtNFIs mutant BKV template with the 1st NFI binding site (located adjacent to the core origin) being wild type (W1) (pUC-5mtNFIsW1-BKV); lane 5, 5mtNFIs mutant BKV template with the second NFI binding site being wild type (W2) (pUC-5mtNFIsW2-BKV); lane 6, 5mtNFIs mutant BKV template with the third NFI binding site being wild type (W3) (pUC-5mtNFIsW3-BKV); lane 7, 5mtNFIs mutant BKV template with the sixth NFI binding site being wild type (W6) (pUC-5mtNFIsW6-BKV). Solid arrow indicates replicated test template; dashed arrow indicate replicated competitor template.
Figure 5. *In vitro* pull-down assays to detect interaction between NFI and BKV Tag. (A). Flag antibody pull-down assays to identify transcription factors interacting with BKV Tag. Lane 1, input of HEK293 cell nuclear extracts (1/100 of total input); lane 2, BKV full-length Tag pulled-down fraction (1/10 of total pull-down); lane 3, mock pull-down fraction in the absence of Tag (1/10 of total pull-down). Density of specific transcription factors binding sites in BKV archetype enhancer is illustrated by number of “+”; “+/−” stands for a partially conserved site; NA stands for not available.
available. (B) Illustration of truncation mutants of BKV Tag; (C) GST affinity pull-down assays to determine interaction of specific Tag truncation mutants with transcription factors. Lane 1, HEK293 nuclear extract input (1/80 of total input); lane 2, GST affinity pull-down fraction control; lane 3, GST-tagged BKT C-terminal region (Cter) pull-down fraction; lane 4, GST-tagged BKT helicase/ATPase domain (HD) pull-down fraction; lane 5, GST-tagged BKV Tag helicase and host range domain (HDHR) pull-down fraction. Loading amount of lane 2-5 is 1/10 of total pull-down.
Figure 6. DNA replication assays in the presence of wild type BKV (pBC-wt-BKV) as a competitor while titrating the transient expression of BKTAg. 25 ng pUC-templates, 100 ng pBC-wt-BKV competitor and increasing amount of pCMV-BKT-Flag were transfected into HK-2 cells with carrier DNA; 48 h P.T. cells were harvested and DNA replication was analyzed by Southern blot. The amount of transfected BKTAg expression vector pCMV-BKT-Flag was increased from 20 ng to up to 900 ng. Three test templates including wild type BKV template (pUC-wt-BKV), enhancer deletion BKV template (pUC-Δen-BKV) and 6mtNFIs BKV template (pUC-6mtNFIs-BKV) are tested as indicated. Autoradiography of replication assays presented in the first three lanes with a longer exposure is shown below the main panel. A solid arrow indicates replicated test template as indicated; a dashed arrow indicate replicated competitor template.
**Figure 7. Monopolymerase assays.** DNA replication of wild type BKV template (pUC-wt-BKV) and NFI binding sites mutant BKV template (pUC-6mtNFIs-BKV) were carried out with high (empty columns, 5 pmol) and low (grey columns, 1 pmol) amounts of purified Pol-primase and with increasing amount of purified NFI-C/CTF1 (0, 1 and 2 pmol) as indicated.
Figure 8. Interaction of NFI with primase. (A) NFI-C/CTF1 and each subunit of Pol-primase were co-expressed in HEK293 cells as indicated: lane 1, mock transfection; lane 2, NFI-C/CTF1 alone; lane 3, NFI-C and p48; lane 4, NFI-C and p58; lane 5, NFI-C and p68; lane 6, NFI-C/CTF1 and p180; the expression of NFI-C/CTF1 and subunits of Pol-primase was detected by Western blotting using anti-T7 tag and anti-HA tag antibodies, respectively; (B) NFI-C/CTF1 and p58 subunits of Pol-primase were co-expressed in HEK293 cells as indicated: lane 1, NFI-C/CTF1 and p58 subunit; lane 2, p58 subunit alone; lane 3, NFI-C/CTF1 alone; lane 4, blank control; Western blotting with specific antibodies are indicated beside each panel. The p58 was detected with rabbit polyclonal anti-p58 antibody; expression of NFI-C/CTF1 was detected with anti-HA antibody; NFκB p65 was used as internal negative control; β-actin was used as loading control.