Activation of early gene transcription in polyomavirus BK by human immunodeficiency virus type 1 Tat

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INTRODUCTION

Polyomavirus BK (BKV) is best known for its role as the aetiological agent responsible for polyomavirus-associated nephropathy, which causes a significant percentage of allograft losses in renal transplant recipients. Although BKV infects up to 90% of the population worldwide during childhood and then remains in a latent state in the kidney. In the context of immunosuppression in kidney transplant patients, reactivation of the viral early promoter (BKV_E) results in production of T antigen, enabling virus replication and transition from latency to the lytic phase, causing polyomavirus-associated nephropathy. Reactivation of BKV can also cause complications such as nephritis, atypical retinitis and haemorrhagic cystitis in AIDS patients. Here, the effects of human immunodeficiency virus type 1 (HIV-1) proteins Tat and Vpr on BKV transcription were investigated and it was demonstrated that Tat dramatically stimulated BKV_E. Site-directed mutagenesis analysis of potential Tat-responsive transcriptional motifs complemented by an electrophoretic mobility shift assay (EMSA) showed that Tat activated BKV_E by inducing binding of the NF-κB p65 subunit to a κB motif near the 3’ end of BKV_E. In addition, a sequence within the 5’ UTR of BKV_E transcripts (BKV_E-TAR) was identified that is identical to the HIV-1 transactivation response (TAR) element. The BKV_E-TAR sequence bound Tat in RNA EMSA assays and deletion of the BKV_E-TAR sequence eliminated Tat transactivation of BKV_E transcription. Thus, Tat positively affected BKV_E transcription by a dual mechanism and this may be important in diseases involving BKV reactivation in AIDS patients.

BKV is a polyomavirus, a family of small DNA tumour viruses, and is closely related to Simian virus 40 and polyomavirus JC (JCV), the aetiological agent of progressive multifocal leukoencephalopathy (Khalili et al., 2004; White & Khalili, 2004, 2005). The genome of BKV comprises a circular dsDNA of approximately 5.2 kb. BKV contains a variable bidirectional promoter/enhancer region of ~500 bp, known as the non-coding control region (NCCR), which, in one direction, controls transcription of the early genes pneumonitis (Cubukcu-Dimopulo et al., 2000), encephalitis (Garavelli & Boldorini, 2002; Gray et al., 2003; Lesprit et al., 2001), atypical retinitis (Hedquist et al., 1999) and disseminated infection involving multiple organs (Bratt et al., 1999; Vallbracht et al., 1993). Furthermore, several studies have shown increased levels of BKV in AIDS patients (Knowles et al., 1999; Markowitz et al., 1993; Pietropaolo et al., 2003). In addition, BKV reactivation correlates with the severity of immunodeficiency (Behzad-Behbahani et al., 2004; Knowles et al., 1999; Markowitz et al., 1993). These observations indicate an interaction between HIV-1 and BKV and suggest that BKV may be an emerging AIDS-associated pathogen.
encoding large T and small t antigens. In the opposite direction, the BKV NCCR initiates transcription of the viral late genes for the viral capsid proteins VP1, VP2 and VP3 and the small auxiliary protein, agnoprotein, after the onset of viral DNA replication. Sequences within the NCCR determine the level of early gene expression and thus are also referred to as the BKV early promoter (BKVE). The replicative phase of BKV infection absolutely requires the viral early protein T antigen, which is a component of the multiprotein viral DNA replication complex. Thus, regulation of T antigen expression by BKVE is a key determinant of the balance between latency and lytic infection.

The NCCR of each BKV strain displays a high degree of variability due to mutations, duplications, deletions and rearrangements (Moens & van Ghelu, 2005; Shah, 1996). Rearrangement of the NCCR can occur in patients or during the propagation of virus in tissue culture and is not well understood. The NCCR of archetype BKV, which predominates in urine and is the transmissible form of the virus, is highly conserved and consists of a true palindrome, two inverted repeats (IR1 and IR2), an approximately 20 bp AT-rich region and several blocks designated by the letters P (68 bp), Q (39 bp), R (63 bp) and S (63 bp). Each BKV strain isolated from patient samples contains a unique arrangement of deleted, duplicated and/or rearranged P-Q-R-S segments. For example, the BKV Dunlop NCCR consists of the arrangement P1–64-P1–7,26–68-P1–64-S1–63 in which the Q and R segments are deleted, P is triplicated and an 18 bp segment is deleted from the middle P segment (Fig. 1a; Moens & Rekvig, 2001). Previous studies using linker analysis and DNase protection assays have identified binding sites for transcription factors NF-1 and Sp1 within the NCCR (Deyerle & Subramani, 1988; Ferguson & Subramani, 1994; Markowitz & Dynan, 1988; Moens & Rekvig, 2001). The NF-1 and Sp1 sites are required for efficient early gene transcription (Deyerle & Subramani, 1988). In addition to NF-1 and Sp1 sites, the BKV Dunlop NCCR contains an AP-1 site at each P block junction, which is not present in the BKV archetype. Recently, we found that co-operative interaction of the NF-κB p65 subunit and C/EBPβ transcription factors potently stimulates BKVE, suggesting that NF-κB signalling is involved in BKV reactivation (Gorrill & Khalili, 2005).

The increase in reported incidences of BKV-related complications, as well as increased levels of detectable BKV in AIDS patients, as discussed above, suggests the possibility of an interaction between HIV-1 and BKV. Therefore, we asked whether HIV-1 transcriptional activator proteins could trans-activate the BKV promoter. Here, we report that HIV-1 Tat positively affects BKVE transcription by a dual mechanism including interaction with NF-κB. This may play a role in diseases involving BKV reactivation in AIDS patients.

**METHODS**

**Plasmids.** BKVE-CAT and BKVE-CAT were made by cloning a DNA fragment corresponding to the NCCR of Dunlop strain BKV into the BamHI site of pBLCAT3 (Luckow & Schutz, 1987), which contains the chloramphenicol acetyltransferase (CAT) reporter gene, in the early (BKVE) or late (BKVL) orientation, respectively. The following plasmids have been described previously: BKVE–ΔxB-CAT (Gorrill & Khalili, 2005), pCMV-Tat, pGST, pGST-Tat, pCMV-Vpr (Sawaya et al., 2000), pCMV-p50, pCMV-p65 (Safak et al., 1999), CMV-IxBz and CMV-IxBzAN (Ansari et al., 2001). BKVE-ATAR-CAT was made by site-directed mutagenesis of the transactivation response element (TAR)-like region of BKVE-CAT using primers 5′-CCAAATGATTATTGCATTACCAACAC-3′ and 5′-GGGGCTAGCCAAATC-3′ and the GeneTailor Site-directed Mutagenesis System (Invitrogen). Plasmid pBL3CAT(−450/+30) contains the HIV-1 long terminal repeat (LTR) and plasmid pBL3CAT(−450/+3) contains the HIV-1 LTR minus the TAR. A PCR fragment corresponding to the 5′ untranslated region (UTR) (nt 1–110) of BKV Dunlop was cloned downstream of the LTR of plasmid pBL3CAT(−450/+3) to generate the plasmid pBL3CAT(−450/+3 +3 BKVE UTR).

For experiments involving the NCCR of the archetypal (WW) strain of BKV (Rubinstein et al., 1987), a plasmid containing the WW NCCR in pBlueScript (Stratagene) was kindly provided by Dr Hans Hirsch (University of Basel, Basel, Switzerland). The WW NCCR was cut out with SacI and recloned into the pGL3-basic luciferase reporter vector (Promega) in the early orientation and designated pWWBKVE-LUC.

**Antibodies.** Rabbit anti-p65 (C-20) and goat anti-p50 (C-19) were from Santa Cruz Biotechnology. Anti-GRB2 was from BD Transduction Laboratories.

**Cell culture, transfection and CAT and luciferase assays.** Cell lines were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS (Life Technologies). For transfection of Vero cells, 3 × 10⁶ cells were transfected with 1-μg reporter plasmid (BKVE-CAT, BKVE–ΔxB-CAT, BKVE-ATAR-CAT or BKVL-CAT) with or without 0.5 μg of the following plasmids as indicated in the figure legends: pCMV-Tat, pCMV-Vpr, pCMV-p50, pCMV-p65, CMV-IxBz or CMV-IxBzAN. The Fenugene 6 transcription reagent (Roche) was used according to the manufacturer’s instructions. At 48 h after transfection, protein was harvested and CAT activity was determined as described previously (Coyle-Rink et al., 2002). CV-1, HeLa and U87-MG cells, transfection was performed as follows. CV-1 cells were transfected with 1-μg BKVE-CAT reporter alone or with 0.5 μg pCMV-Tat using Fenugene6. HeLa and U87-MG cells were transfected with 3 μg BKVE-CAT reporter alone or in combination with 0.5 μg pCMV-Tat using the calcium phosphate precipitation method (Graham & van der Eb, 1973). CAT and luciferase assays were performed using assay kits (Promega) according to the protocols provided by the manufacturer.

**Primer extension analysis.** Primer extension analysis to identify the BKVE transcription initiation site directed by HIV-1 Tat was performed as follows. CV-1 cells (1 × 10⁶) were seeded on to 100 mm dishes. After 24 h, cells were transfected with 1 μg BKVE-CAT reporter alone or in combination with 0.5 μg pCMV-Tat. At 48 h after transfection, total RNA was isolated using the TRIzol reagent (Invitrogen). Total RNA (50 μg) was hybridized with 10⁶ c.p.m. 5′-labelled primer (5′-TCCAGTGTATTTTTCTCCCAT-3′, which anneals to the 5′ end of the CAT gene) overnight in hybridization buffer [400 mM NaCl, 40 mM PIPES/NaOH (pH 6.4), 1 mM EDTA (pH 8) and 80% formamide (pH 6.1)]. Hybridized samples were precipitated and incubated in a reaction containing 50 U avian myeloblastosis virus reverse transcriptase (Roche) at 42°C for 40 min in 50 mM Tris/HCl (pH 8), 5 mM MgCl₂, 5 mM DTT, 50 mM KCl, 50 mg BSA ml⁻¹ and 160 mM each dNTP. Samples were extracted with phenol/chloroform and resolved on a 6% polyacrylamide gel.

**In vivo and in vitro production of proteins.** GST and GST–Tat fusion protein were prokaryotically expressed and purified as follows.
Fig. 1. Transcriptional regulation of the BKV E promoter by HIV-1 Tat. (a) The NCCR of BKV Dunlop strain depicting the palindrome (Pal), two inverted repeats (IR1 and IR2), the 20 bp AT-rich region (A/T), the three direct repeats (P) and a single 63 bp block (S) (Moenes & van Ghelue, 2005). The arrow above the figure indicates the major early transcription start site. Nucleotide 1 is the start of the T-antigen coding region. The position of the xB site and BKV E-TAR sequence are indicated. (b) Effect of HIV-1 Tat and Vpr on the transcriptional activity of the BKV E and BKV L promoters in Vero cells. Vero cells were transfected with the BKV E-CAT or BKV L-CAT reporter plasmid either alone or in combination with pCMV-Tat or pCMV-Vpr, the proteins were harvested and CAT activity was determined as described in Methods. The results shown represent two separate transfection experiments and the numbers indicate fold activity ± SD. (c) Effect of Tat on the transcriptional activity of the BKV E promoter in CV-1, HeLa and U87-MG cells. Cells were transfected with BKV E-CAT with or without pCMV-Tat. The results shown are from two separate transfection experiments and numbers indicate fold activation ± SD. (d) Primer extension analysis to identify the BKV E transcription initiation site directed by HIV-1 Tat. CV-1 cells were transfected with BKV E-CAT with or without pCMV-Tat and total RNA was isolated and hybridized with a primer that anneals to the 5’ end of the CAT gene. Hybridized samples were precipitated, incubated with reverse transcriptase and resolved on a polyacrylamide gel. The region from the BKV E promoter that corresponds to the primer extension is depicted schematically to the right of the autoradiogram with nt 88–98 of the BKV E genome shown in the expansion. G* indicates nt 93, which corresponds to the start site of BKV E transcription upon Tat induction. An A/G sequence ladder, which was run on the same gel and used to determine the size of the primer extension product, is shown in the right-hand panel. (e) Effect of HIV-1 Tat on the transcriptional activity of a promoter derived from the NCCR of the archetypal strain (WW) of BKV in Vero cells. Vero cells were transfected with pWWBKVE-LUC reporter plasmid with or without pCMV-Tat, proteins were harvested and luciferase activity was determined as described in Methods.
Overnight cultures of bacteria expressing recombinant GST–Tat or GST alone were diluted 10-fold. Cultures were grown at 37 °C to an OD600 of 0.6 and induced to express GST fusion protein by adding 0.1 mM IPTG for 3–4 h. Cells were pelleted by centrifugation and resuspended in EBC-DTT buffer [50 mM Tris/HCl (pH 8), 120 mM NaCl, 0.5% IGEPAL and 5 mM DTT]. After brief sonication and centrifugation, the supernatant was incubated with glutathione-Sepharose beads (Amersham Biosciences) at 4 °C for 30 min. Beads were washed extensively with fresh EBC-DTT buffer and GST fusion protein was collected by incubation in GST elution buffer [100 mM Tris/HCl, 2 mM DTT, 20 mM free glutathione (Sigma)]. The integrity and purity of the GST fusion protein was analysed by SDS-PAGE followed by Coomassie blue staining. Known amounts of BSA were included on the same gel for determination of the yield of the full-length protein.

**Treatment of HL3T1 cells with GST and GST–Tat.** HL3T1 cells (a stable HeLa-derived cell line that contains several integrated copies of the CAT gene under the control of the HIV-1 LTR) were treated with GST or GST–Tat protein as follows. HL3T1 cells (3 × 10⁶) were plated on 60 mm dishes. After 24 h, cells were transfected with GST–Tat or GST. Transfections were prepared by the method of Demarchi et al. (1996). GST or GST–Tat (5 μg) was combined with 300 μl OptiMEM medium (Invitrogen). Similarly, 25 μl lipofectin (Invitrogen) was combined with 300 μl OptiMEM medium. Both mixtures were combined and incubated at room temperature for 10 min. HL3T1 cells were rinsed once with OptiMEM and 2-4 ml OptiMEM was added to the cells. After the 10 min incubation, lipo-fection/GST protein mixtures were added to the cells. After 4 h, the OptiMEM/transfection mixture was replaced with fresh DMEM with 10% FBS. At 24 h post-transfection, protein was collected and CAT activity was determined.

**Electrophoretic mobility shift assay (EMSA).** An EMSA was performed as follows to assess the binding of NF-κB to the BKV κB motif in response to Tat. HeLa cells (5 × 10⁶) were seeded on 100 mm dishes. After 24 h, cells were transfected with GST or GST–Tat as described above. At 5 h post-transfection, nuclear extracts were collected according to the method of Andrews & Faller (1991). As a positive control for activation of NF-κB, HeLa cells were treated with 100 ng phorbol 12-myristate 13-acetate (PMA; Sigma) ml⁻¹ for 30 min. Nuclear extract (10 μg) from untreated, PMA-treated, GST-treated and GST–Tat–treated cells was incubated with 50 000 c.p.m. of a 32P-labelled double-stranded oligonucleotide probe containing the BKV κB motif (5′-TTCGTTAATTTGCGAAATAGGGATGCCCAATAA-3′), as described previously (Safak et al., 1999).

**RNA interference with p65 small interfering (si) RNA.** Transient knock-down of p65 was performed with an siRNA specific for p65 (5′-GCCCUCACCCUUACGUCADTD-3′; Dharmacon Research).

Cells (3 × 10⁶) were plated on 60 mm dishes. After 24 h, cells were rinsed once with OptiMEM. siRNA was added to a final concentration of 50 nM by the method of Surabhi & Gaynor (2002). At 24 h after siRNA transfection, cells were transfected as described above and CAT activity was determined. Western blot analysis of protein extracts from untransfected cells or cells transfected with p65-specific siRNA using anti-p65 was used to ascertain p65 knock-down, with anti-Grb2 antibody used as a loading control. Control non-targeting siRNA was also obtained from Dharmacon.

**Tat RNA EMSA.** The oligoribonucleotide 5′-GUGGAGGCUCUUUCUGAGGCCACG-3′ (BKV TAR), which corresponds to nt 70–46 of the BKV genome, was end-labelled using [32P]ATP with T4 polynucleotide kinase. The labelled BKV TAR was incubated in a reaction mixture with recombinant Tat using the method of Wei et al. (1998). To obtain recombinant Tat, Tat was cleaved from GST beads by cleavage in buffer [50 mM Tris/HCl (pH 7.6), 20 mM KCl, 1 mM DTT] containing thrombin.

**PMA treatment.** For experiments to measure the effect of PMA on BKV E transcription, cells were transfected as described above and treated 24 h later with 100 ng PMA ml⁻¹. After a further 24 h, cells were harvested for the CAT assay.

**RESULTS**

To investigate whether HIV-1 transcriptional regulatory proteins could transactivate the BKV promoter, a DNA fragment corresponding to the NCCR of the Dunlop strain of BKV (Fig. 1a) was fused in the early (BKVE) or late (BKVL) orientation into the BamHI site of pBLCAT3 to give the BKVE-CAT and BKVL-CAT reporter plasmids, respectively. These constructs were introduced into the Vero monkey kidney cell line, alone or in combination with expression constructs for the HIV-1 proteins Vpr and Tat, and the transcriptional activity was determined. As can be seen in Fig. 1(b), expression of Tat significantly activated transcription from the BKVE promoter. Neither Vpr nor Tat had a significant effect on the BKVL promoter. The effect of Tat on BKVE transcriptional activity was also evident in several other cell lines (Fig. 1c), suggesting that the observed event was not cell-type specific. Since others have shown that the BKVE transcription initiation site may vary (Moens & Rekvig, 2001), we next determined the site of Tat-induced transcription initiation. Vero cells were transfected with the BKVE-CAT reporter alone or in combination with the Tat expression construct and the transcription initiation site was determined by primer extension analysis by annealing total RNA to a primer specific for the 5′ end of the CAT gene. As shown by an arrow in Fig. 1(d), the initiation site for Tat-directed transcription was located at nt 93, which has been reported by others to be the major transcription start site for BKV transcription (Deyerle et al., 1987; Ferguson & Subramani, 1994; Moens & Rekvig, 2001; Seif et al., 1979). An A/G sequence ladder was run on the same gel and used to determine the size of the primer extension product (Fig. 1d).

In order to determine whether the observed Tat stimulation was peculiar to the Dunlop strain of BKV or might be a general feature of the virus, we constructed a reporter plasmid in which the archetypal, non-rearranged form of the BKV NCCR (WW) drives luciferase expression in the early orientation (pWWBKVET-LUC). Tat significantly activated transcription from the archetypal BKVE promoter (Fig. 1e). All subsequent experiments were performed with the Dunlop promoter.

The BKVE promoter has a κB motif downstream from the transcription initiation site at nt 25–34 (shown in Fig. 1a). As the HIV-1 Tat protein has been shown to activate NF-κB-dependent transcription (Demarchi et al., 1996), we investigated next whether Tat transactivation of the BKVE promoter depended on this site. Vero cells were transfected with the BKVE reporter alone or with the
indicated combinations of expression constructs for Tat, IxBz or IxBzΔN. IxBz is an NF-κB-binding protein that sequesters NF-κB in the cytoplasm until IxBz becomes phosphorylated by an upstream signalling kinase and is degraded by the ubiquitin-proteasome pathway, thus releasing active NF-κB to the nucleus. IxBzΔN is an N-terminally truncated mutant of IxBz that lacks two serine residues that are phosphorylated upon activation of the NF-κB pathway and are required for degradation via the ubiquitin-proteasome pathway (Ghoda et al., 1997). Whereas IxBz had no effect on Tat-dependent BKV_E transcription, co-expression of IxBzΔN with Tat abolished the Tat-dependent BKV_E transcriptional response (Fig. 2a). Similarly, site-directed mutagenesis of the BKV_E-κB motif (BKV_E-ΔκB-CAT) resulted in loss of Tat responsiveness (Fig. 2b, lanes 3 and 4) compared with the wild-type promoter (Fig. 2b, lanes 1 and 2).

Based on these results, we concluded that induction of the BKV_E promoter by Tat is NF-κB-dependent. Furthermore, since Tat does not bind DNA, we sought to determine whether Tat induced the binding of NF-κB to its cognate site. To this end, we employed EMSA using Tat protein and the κB DNA motif as a probe. The biological activity of the recombinant GST–Tat fusion protein used in this experiment was confirmed as follows. Transient assays were performed by treating HL3T1 cells, a HeLa-cell derivative that contains multiple integrated copies of the CAT gene under control of the HIV-1 LTR, with GST or GST–Tat. As shown in Fig. 3(a), treatment of HL3T1 cells with GST–Tat, but not GST, caused a dramatic induction of CAT activity, demonstrating that the GST–Tat preparation was biologically active. Next, nuclear extracts prepared from HeLa cells treated with GST (Fig. 3b, lanes 4–8) or GST–Tat (Fig. 3b, lanes 9–13) were incubated with a double-stranded

**Fig. 2.** Involvement of NF-κB in Tat-dependent transactivation of BKV_E transcription. (a) Vero cells were transfected with BKV_E-CAT with or without the indicated combinations of pCMV-Tat, CMV-IxBz and CMV-IxBzΔN. Results are from two separate transfection experiments and numbers indicate fold activation ± SD. (b) Analysis of requirement for the NF-κB motif in the BKV_E promoter for Tat-dependent transcriptional activation. Vero cells were transfected with BKV_E-CAT or BKV_E-ΔκB-CAT reporter, where the NF-κB site had been removed by site-directed mutagenesis, with or without pCMV-Tat. Results are from two separate transfection experiments and numbers indicate fold activation ± SD. Histograms were normalized to lane 1 (wild-type untreated promoter).

**Fig. 3.** HIV-1 Tat induces the binding of NF-κB p65 to the BKV_E κB motif. (a) Transcriptional activity of the HIV-1 LTR was measured following treatment of HL3T1 cells with recombinant HIV-1 Tat protein. To assess the biological activity of GST–Tat, HL3T1 cells were transfected with GST–Tat or GST and CAT activity was determined. (b) EMSA to assess the binding of NF-κB to the BKV_E κB motif in response to Tat. HeLa cells were transfected with GST or GST–Tat as described in Methods and nuclear extracts were collected. As a positive control for activation of NF-κB, HeLa cells were treated with PMA for 30 min. Nuclear extract from untreated (lane 2), PMA-treated (lane 3), GST-treated (lanes 4–8) and GST–Tat-treated (lanes 9–13) cells were incubated with probe containing the BKV_E κB motif. Lane 1 contained probe only. GS, Goat serum; RS, rabbit serum.
32P-labelled oligonucleotide probe containing the BKV\textsubscript{E} \(\kappa B\) motif. The presence of the p50 and/or p65 subunits of NF-\(\kappa B\) in the protein–DNA complexes was determined with the use of anti-p50 (Fig. 3b, lanes 7 and 12) or anti-p65 (Fig. 3b, lanes 8 and 13) antibodies. Nuclear extract from PMA-treated HeLa cells was included as a positive control for NF-\(\kappa B\) binding activity (Fig. 3b, lane 3). In contrast to extracts from untreated and GST-treated cells (Fig. 3b, lanes 2 and 4–8, respectively), GST–Tat induced the binding of a band that co-migrated with extract from PMA-treated cells (Fig. 3b, compare lane 9 with lane 3). This band disappeared with p65-specific antibody (Fig. 3b, lane 13), but not with control sera (Fig. 3b, lanes 10 and 11) or with p50-specific antibody (lane 12), demonstrating the presence of p65 in the complex.

To investigate further the involvement of p65 in the Tat-dependent activation of BKV\textsubscript{E} transcription, we transfected Vero cells with the BKV\textsubscript{E} reporter alone or with the indicated combinations of expression vectors for the p50 and p65 subunits of NF-\(\kappa B\) and the transcriptional activity was determined (Fig. 4a). In agreement with the EMSA results, overexpressed p65, but not p50, dramatically induced BKV\textsubscript{E} promoter activity. Furthermore, co-expression of p65 and p50 gave results similar to p65 alone. Next, we used siRNA transiently to knock down the expression of the p65 subunit of NF-\(\kappa B\) and repeated the transfection experiment with BKV\textsubscript{E} reporter alone or in combination with overexpressed Tat. Transient siRNA-dependent knock-down of p65 in HeLa cells significantly reduced Tat-dependent induction of BKV\textsubscript{E} promoter activity, indicating the importance of endogenous p65 in Tat stimulation of BKV\textsubscript{E} (Fig. 4b). The efficacy of siRNA for the suppression of p65 protein level was tested by Western blot analysis (Fig. 4c). Non-specific siRNA did not suppress the level of p65 protein, nor did it affect the rate of BKV\textsubscript{E} transcription significantly.

Upon careful analysis of the BKV\textsubscript{E} promoter for potential Tat-responsive sequences, we also noticed the pentanucleotide 5\textsuperscript{‘}-\textsuperscript{3\textsuperscript{‘}}TCAGA-3\textsuperscript{‘}, which, when transcribed in the direction of early transcription, encodes the RNA sequence 5\textsuperscript{‘}-UCUGA-3\textsuperscript{‘}, the same sequence that is present in the bulge of the HIV-1 TAR RNA to which Tat binds to stimulate efficient elongation of HIV-1 RNA transcripts (Fig. 1a; Rounseville & Kumar, 1992). Next, we sought to determine the importance of this sequence, which we designated BKV\textsubscript{E}–TAR, for Tat transactivation of the BKV\textsubscript{E} promoter. Hence, we deleted the BKV\textsubscript{E}–TAR pentanucleotide by site-directed mutagenesis to give the construct BKV\textsubscript{E}–D\textsubscript{TAR} and tested for responsiveness to Tat. Vero cells were transfected with wild-type BKV\textsubscript{E} or the BKV\textsubscript{E}–TAR construct alone or in combination with the Tat expression construct and the transcriptional activities were compared (Fig. 5a). Whereas Tat activated the wild-type BKV\textsubscript{E} reporter, mutation of the BKV\textsubscript{E}–TAR site extinguished Tat responsiveness, demonstrating the requirement of the TAR-like site for Tat transactivation of the BKV\textsubscript{E} promoter. We also performed an RNA EMSA using a 32P-labelled RNA oligonucleotide containing the pentanucleotide BKV\textsubscript{E}–D\textsubscript{TAR} plus flanking sequences. Recombinant GST–Tat was cleaved from GST beads with thrombin (Fig. 5c). Incubation of cleaved Tat with the BKV\textsubscript{E}–TAR probe resulted in a retarded band.

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**Fig. 4.** Involvement of the p65 subunit of NF-\(\kappa B\) in transactivation of the BKV\textsubscript{E} promoter by HIV-1 Tat. (a) Vero cells were transfected with the BKV\textsubscript{E}–CAT reporter with or without pCMV-p50, pCMV-p65 or both, and CAT activity was determined. Numbers indicate fold activation ± SD. (b) Effect of transient knock-down of p65 using RNA interference with a p65-specific siRNA. HeLa cells were transfected with p65-specific or non-specific (ns) siRNA, transfected with plasmid and CAT activity was determined. (c) Western blot of proteins from cells that were not transfected (lane 1) or were transfected with non-specific (lane 2) or p65-specific siRNA (lane 3) using anti-p65 and anti-Grb2 antibodies.
(Fig. 5b), demonstrating that Tat binds to BKV_E-TAR. Hence, we concluded that Tat also requires the BKV TAR sequence for transactivation of the BKV_E promoter.

To investigate whether the BKV TAR element could function in a heterologous promoter, we replaced the TAR of the HIV-1 LTR with the 5' 3' UTR from the BKV_E promoter. Full-length HIV-1 LTR is responsive to Tat, but this is lost when the HIV-1 TAR element is deleted. However, the BKV TAR sequence failed to rescue Tat-responsiveness of the TAR-less HIV-1 LTR (Fig. 6).

Finally, we performed an analysis to compare all three promoters (wild-type BKV_E, ΔxB and ΔTAR) for the effect of PMA stimulation, which activates NF-κB, and the relative effect of expression of p65 or Tat (Fig. 7). PMA stimulated the wild-type BKV_E promoter 3-2-fold (Fig. 7, compare lanes 1 and 2). The basal levels of activity of both the ΔxB and ΔTAR mutant promoters were lower than the wild-type BKV_E promoter by 0-28-fold and 0-43-fold, respectively (Fig. 7, compare lanes 1, 5 and 9). The activity of these promoters in the presence of PMA was also much reduced (Fig. 7, compare lanes 2, 6 and 10) and no statistically significant difference was seen for the ΔxB promoter in the presence and absence of PMA (Fig. 7, compare lanes 5 and 6). Both of the mutant promoters were unresponsive to Tat (Fig. 7, compare lane 5 with lane 8 and lane 9 with lane 12), as was also shown in Figs 2 and 5. The ΔTAR but not the ΔxB promoter was responsive to p65 expression (compare lane 9 with lane 11 and lane 5 with lane 7).

**DISCUSSION**

The results presented in this study demonstrate the ability of HIV-1 Tat to transactivate the BKV_E promoter in several cell

**Fig. 5.** HIV-1 Tat binds to the early leader RNA of BKV_E transcripts. (a) Analysis of the dependence of Tat-dependent BKV_E transcriptional activation on the TAR-like motif in the BKV_E promoter. Vero cells were transfected with the BKV_E or BKV_E-ΔTAR reporter construct (where the TAR-like region of BKV_E-CAT had been deleted by site-directed mutagenesis) with or without pCMV-Tat as described in Methods and CAT activity was determined. Results are from two separate transfection experiments ± SD. Histograms were normalized to lane 1 (wild-type untreated promoter). (b) Tat RNA EMSA. A BKV TAR oligoribonucleotide (nt 70–46 of the BKV genome) was end-labelled and incubated with recombinant Tat. Lane 1, probe only; 2, 100 ng Tat. The positions of the probe and the Tat–RNA complex are indicated. (c) Coomassie blue-stained SDS-polyacrylamide gel with 2 μg cleaved Tat. The position of the cleaved Tat is indicated.

**Fig. 6.** The BKV TAR sequence fails to rescue Tat responsiveness of the TAR-deleted HIV-1 LTR. HeLa cells were transfected with three HIV-1 LTR–CAT reporter constructs as indicated with or without pCMV-Tat and CAT activity was determined. Reporter plasmid constructs were as follows: LTR-CAT −450/+80 is pBL3CAT(−450/+80), which contains the full-length HIV-1 LTR (numbering is relative to the start site of transcription). LTR-CAT −450/+3 is pBL3CAT(−450/+3), which contains the HIV-1 LTR minus the TAR element located in region −3 to +80. LTR-CAT/BKV5'UTR is pBL3CAT(−450/+3+BKV5'UTR), which contains BKV 5' UTR downstream of the LTR of pBL3CAT(−450/+3), i.e. the BKV TAR element replaces HIV-1 TAR.
Furthermore, it is well documented that, in the absence of the TAR region, Tat can activate transcription of the HIV-1 LTR, as well as several heterologous promoters, through its association with NF-κB (Biswas et al., 1995; Demarchi et al., 1996). For example, Tat directs E-selectin expression in human umbilical vein endothelial cells by inducing the binding of NF-κB to a κB motif in the E-selectin promoter (Cota-Gomez et al., 2002). In this study, we have demonstrated likewise the requirement for a κB motif for initiation of transcription from the BKV E promoter by Tat.

In vivo, there are several modes of action by which Tat potentially could activate the BKV E promoter and cause BKV reactivation. First, biologically active Tat is secreted by HIV-1-infected cells and could affect neighbouring cells by transcellular means or by binding to cell-surface receptors (reviewed by Peruzzi, 2006). Alternatively, if an HIV-1-infected cell becomes superinfected with BKV or vice versa, newly synthesized Tat could activate BKV E transcription, as has been shown to occur for transactivation of Human herpesvirus 5 (human cytomegalovirus) (Ho et al., 1991; Skolnik et al., 1988). From the dual requirement for NF-κB and BKV TAR binding in the present study, we can propose a model in which Tat first initiates transcription by inducing the binding of NF-κB p65 to the BKV E promoter. Subsequently, Tat may bind to the 5’ UTR of BKV E transcripts and enhance transcriptional activation through an HIV-1 TAR-like mechanism. Furthermore, when we replaced the TAR of the HIV-1 LTR with the 5’ UTR from the BKV E promoter, the BKV TAR sequence failed to rescue Tat responsiveness of the TAR-less HIV-1 LTR (Fig. 6). Therefore, the possibility of only a TAR-like mechanism for transcriptional activation of the BKV E promoter in response to Tat is probably an oversimplification. It may be that BKV TAR-bound Tat must also interact either directly or indirectly with promoter-bound NF-κB p65 to activate BKV E transcription.

As regards the mechanism of NF-κB activation by Tat, this has been the subject of intensive investigation in this laboratory and others. It has become clear that this activation can occur by both direct and indirect mechanisms. Direct interaction with NF-κB is involved in TAR-independent activation of the HIV-1 LTR in cells from the CNS (Taylor et al., 1995) and may involve a third protein named NFBP (Sweet et al., 2005). As regards indirect interaction, it has been demonstrated previously, by ourselves and others, that Tat induces the expression of TNF-α (Buonaguro et al., 1992; Darbinian et al., 2001; Rautonen et al., 1994; Sawaya et al., 1998), which can activate the NF-κB pathway (reviewed by Chen & Goeddel, 2002). Thus, there exist direct and indirect mechanisms for the induction of NF-κB by Tat. As we report here for the BKV NCCR, it has also been reported that NF-κB and Tat act synergistically to increase transcription from the HIV-1 LTR (Nabel & Baltimore, 1987; West et al., 2001).

In conclusion, the data presented in this communication provide evidence for HIV-1 action on the BKV E promoter that complements clinical data in the literature that indicate

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**Fig. 7.** PMA stimulation of wild-type and mutant BKV E promoters. Vero cells were transfected with BKV reporter plasmids (BKVE-CAT, BKVE-ΔκB-CAT or BKVE-ΔTAR-CAT as indicated) together with plasmids expressing p65 or Tat. After 24 h, cells were left untreated or were treated with 100 ng PMA ml⁻¹. After a further 24 h, cells were harvested and CAT activity was determined. The histogram was normalized to lane 1 (untreated wild-type BKV E promoter alone).
an emerging role for BKV in AIDS pathology and point to the importance of a role for HIV-1 Tat in BKV reactivation in AIDS patients.

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