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Kaposi's sarcoma-associated herpesvirus K8 protein interacts with hSNF5

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Kaposi's sarcoma-associated herpesvirus (KSHV) is a human gammaherpesvirus related to Epstein–Barr virus (EBV) and herpesvirus saimiri. KSHV open reading frame K8 encodes a basic region-leucine zipper protein of 237 aa that homodimerizes. K8 shows significant similarity to the EBV immediate-early protein Zta, a key regulator of EBV reactivation and replication. In this study, a carboxyl-terminal deletion mutant of K8, K8(1–115), that had strong transactivating properties was found. Screening using transcriptionally inactive K8(1–75) showed that K8 interacts and co-localizes with hSNF5, a cellular chromatin-remodelling factor, both *in vivo* and *in vitro*. This interaction requires aa 48–183 of hSNF5 and 1–75 of K8. In a yeast expression system, the ability of K8 and K8(1–115) to activate transcription requires the presence of SNF5, the yeast homologue of hSNF5. These data suggest a mechanism by which the SWI–SNF complex is recruited to specific genes. They also suggest that K8 functions as a transcriptional activator under specific conditions and that its transactivation activity requires its interaction with the cellular chromatin remodelling factor hSNF5.

INTRODUCTION

Kaposi's sarcoma-associated herpesvirus (KSHV), or human herpesvirus-8 (HHV-8), is a gammaherpesvirus related to Epstein–Barr virus (EBV) and herpesvirus saimiri (Boshoff & Weiss, 1998; Russo *et al.*, 1996; Schulz *et al.*, 1998). KSHV is implicated in the pathogenesis of Kaposi's sarcoma (KS), primary effusion B cell lymphomas and multicentric Castleman's disease. Like EBV, KSHV infection in tumour tissue or lymphoma-derived cell lines is predominantly latent. The KSHV genome is present in a latent state in B cell lines, such as body cavity-based lymphoma cells (BCBL-1). These cells have multiple copies of circularized KSHV DNA maintained in the nucleus as episomes whose expression is highly attenuated (Boshoff & Weiss, 1998; Cesarman *et al.*, 1995; Soulier *et al.*, 1995).

The most important step in the KSHV life cycle may be the switch from latency to lytic replication; virus lytic replication is critical for the development of KS (Boshoff *et al.*, 1995; Miller *et al.*, 1997; Schulz *et al.*, 1998). In the latent phase, expression of a limited number of viral genes occurs and the genome is maintained in an episomal form. Lytic phase is characterized by a cascade of gene expression resulting in productive release of virions from infected cells.

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Lytic reactivation of KSHV can be induced artificially by phorbol esters such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) or *n*-butyrate, in a manner similar to EBV-infected B cell lines (Renne *et al.*, 1996). Upon chemical induction, KSHV produces immediate-early viral transcripts within 4 h. These transcripts encode viral transcriptional activator proteins, such as open reading frame 50 (ORF 50). From its expression pattern and function, ORF 50 appears to be the principal driver of the lytic cascade and functions as a switch gene in the disruption of latency (Lukac *et al.*, 1998; Sun *et al.*, 1998; Zhu *et al.*, 1999).

The KSHV ORF K8 gene encodes an early lytic protein that is activated by, and expressed after, KSHV ORF 50 protein (Lin *et al.*, 1999; Sun *et al.*, 1999). Three forms of K8 protein result from alternative splicing and usage of different stop codons. The major form of K8 is a protein of 237 aa with a prototypic basic region-leucine zipper (bZIP) domain at the carboxyl terminus and it homodimerizes using this domain. An acidic domain between residues 6 and 47 (Zhu *et al.*, 1999) suggests that K8 (K-bZIP) may be a member of the bZIP family of transcription factors. This hypothesis is supported further by amino acid sequence analysis showing significant similarity between K8 and the EBV immediate-early gene product Zta (BZLF1, EB1, Z), a transactivator responsible for EBV replication and reactivation from latency to the lytic life cycle (Gruffat *et al.*, 1999; Lin *et al.*, 1999; Zhu *et al.*, 1999).

Recent data showed that K8 homodimerizes in the cytoplasm and is transported to the nucleus (Hwang *et al.*, 2001; Portes-Sentis *et al.*, 2001). K8 interacts and co-localizes with CREB-binding protein (CBP) and has the capacity to modulate CBP-mediated transcription (Hwang *et al.*, 2001). K8 also represses the transcriptional activity of p53 (Park *et al.*, 2000). These data imply that K8 may be a nuclear protein involved in KSHV-driven transcription. K8 associates and co-localizes with the KSHV pseudo-replication compartment structure and with the promyelocytic leukaemia protein (PML) in PML oncogenic domains (PODs) (Wu *et al.*, 2001). It is known that p53 is sequestered in virus replication centres in the nuclei of cells infected with human cytomegalovirus (Fortunato & Spector, 1998) and that K8 recruits p53 to the PODs (Katano *et al.*, 2001). PML is associated with virus replication (Adamson & Kenney, 2001; Bell *et al.*, 2000), suggesting further that K8 plays a role in KSHV replication. Finally, K8 is phosphorylated by cyclin-dependent kinases (CDKs) and the phosphorylation state of K8 may link KSHV DNA replication to the cell cycle (Polson *et al.*, 2001).

In the present study, we show that K8 interacts with hSNF5, a component of the SWI–SNF chromatin-remodelling complex. Yeast two-hybrid screening showed that K8 binds to hSNF5 and further experiments showed this interaction requires aa 48–183 of hSNF5 and 1–75 of K8. In mammalian BCBL-1 cells, which contain KSHV DNA, K8 co-immunoprecipitates with hSNF5. We found that K8 and mutant K8(1–115) have transactivating properties and in yeast these required SNF5, the yeast homologue of hSNF5. We propose that K8 functions as a transcriptional activator and that this activity requires interaction with the cellular chromatin-remodelling factor hSNF5.

METHODS

Cells. 293T cell lines were used for immunoprecipitation, immunohistochemistry and reporter assays. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10 % foetal bovine serum (FBS). For immunoprecipitation, primary effusion lymphoma BCBL-1 cell lines were also used. BCBL-1 cell lines are negative for both human immunodeficiency virus (HIV) and EBV but positive for KSHV. BCBL-1 cells were cultured in RPMI medium supplemented with 10 % FBS. All culture media contained penicillin/streptomycin (50 U ml^{-1}) and all cells were grown in humidified 5 % CO_2 at 37 °C.

Plasmids. Full-length K8 and its deletion mutants (together referred to as the 'K8 series') were cloned into the pcDNA3 plasmid (Invitrogen), as described previously (Hwang *et al.*, 2001), to create the pcDNA3/K8 series. To construct hybrid expression vectors containing the LexA DNA-binding domain (DBD), we subcloned *Eco*RI/*Xho*I-digested fragments of the pcDNA3/K8 series into the *Eco*RI–*Xho*I site of pLexA (Clontech). For Gal4–DBD-fused expression vectors, *Eco*RI–*Xho*I fragments of the pcDNA3/K8 series were subcloned into the *Eco*RI–*Sal*I site of pM (Clontech), and *Eco*RI–*Xba*I fragments of the pcDNA3/K8 series were subcloned into the *Eco*RI–*Xba*I site of pcDNA3-Gal4 (Gal4–DBD is inserted in pcDNA3). Glutathione *S*-transferase (GST)-fused mammalian expression vector pEBG/K8 was constructed by subcloning K8 products amplified by PCR from the pcDNA3/K8 series into the *Bam*HI–*Not*I site of pEBG (a gift from J. Jung, Harvard Medical School, MA, USA). To construct amino-terminal, Flag-tagged vectors, we subcloned *Eco*RI–*Xho*I fragments of the pcDNA3/K8 series into the *Eco*RI–*Xho*I site of pME18S [an SRα promoter-based eukaryotic expression vector (Shiio *et al.*, 1992)]. To construct green fluorescent protein (GFP)-tagged K8, the K8 PCR product from pcDNA3/K8 was subcloned into the *Eco*RI–*Xho*I site of pEGFP-C1 (Clontech) to create pEGFP/K8.

To construct the Flag-tagged and prokaryotic GST-fused hSNF5 expression vector, *Eco*RI/*Xho*I-digested fragments of the hSNF5 PCR product from pcDNA-HA/hSNF5 were subcloned into the *Eco*RI–*Xho*I sites of pME18S and pGEX4T-1 (Amersham Pharmacia), respectively. pEBG/hSNF5 was constructed by subcloning *Bam*HI–*Not*I fragments of pGEX4T-1/hSNF5 into the *Bam*HI–*Not*I site of pEBG.

Yeast two-hybrid assay. The yeast two-hybrid assay was performed using the MATCHMAKER LexA Two-Hybrid system, according to the manufacturer's instructions (Clontech). The yeast strain EGY48 (*MATα his3 trp1 ura3 6lex*Aop-*LEU2*) harbouring pLexA/K8(1–75) and p8op-lacZ was transformed (by the lithium acetate method) with a B cell cDNA fusion library (20 μg) cloned into the activation-domain vector pB42AD. Transformants were selected by culture on synthetic minimum medium (−His/−Leu/−Trp/−Ura) with galactose for 3 days and were then patched onto fresh synthetic minimum medium (−His/−Trp/−Ura) with glucose. Positive clones were tested for galactose-dependent β-galactosidase activity. β-Galactosidase expression in yeast was assayed as described previously (Guarante, 1983).

Transfection and reporter assay. 293T cells were transfected using the calcium phosphate precipitation method (Graham & van der Eb, 1973) and 48 h later were washed with PBS and lysed (Promega Cell Culture Lysis reagent). Reporter plasmid luciferase activity was measured according to the manufacturer's instructions (Promega) and was calculated after normalizing to β-galactosidase activity from a co-transfected Rous sarcoma virus (RSV)-β-Gal control plasmid. All experiments were performed in triplicate and the expression of each plasmid was verified by Western blot assay. DNA masses used for transfections were 1 μg reporter plasmid, 100 ng RSV-β-Gal control plasmid and 1 μg expression plasmid. The total amount of expression vector was kept constant.

Immunoblot analysis. Transfected 293T cells or transformed yeast cells were washed with PBS and lysed with 6 × SDS sample buffer [0·28 M Tris/HCl, pH 6·8, 30 % (v/v) glycerol, 1 % (w/v) SDS, 0·5M DTT and 0·0012 % (w/v) bromophenol blue]. Cell lysates were subjected to SDS-PAGE and electroblotted onto nitrocellulose membranes. After blocking with 5 % skimmed milk, membranes were incubated with appropriate first antibody (α-Gal4, α-Flag, α-GST, α-K8, α-hSNF5, α-LexA or α-β-Actin) at a concentration of 100 ng ml^{-1} for 1 h, then appropriate secondary antibody (anti-mouse or anti-rabbit) conjugated with alkaline phosphatase for 1 h at a concentration of 50 ng ml^{-1}. All procedures were performed at room temperature and membranes were washed with PBST (0·1 % Tween 20 in PBS) between each step. The reaction product was visualized using Enhanced Chemiluminescence (ECL) reagent, according to the manufacturer's instructions (Amersham Pharmacia).

Immunoprecipitation. Transfected 293T or TPA-stimulated BCBL-1 cells were washed with PBS and lysed 48 h after transfection or chemical induction. Cells were harvested and lysed in binding buffer [20 mM HEPES (pH 7·4), 100 mM NaCl, 1 % Triton X-100, 0·5 % NP-40 and protease inhibitors], mixed for 1 h at 4 °C and centrifuged to remove cell debris. After pre-clearing by absorption to protein G- and A-Sepharose (Santa Cruz Biotechnology), lysates were incubated with antibodies against Flag (α-Flag), GST (α-GST), K8 (α-K8) or hSNF5 (α-hSNF5) at a concentration of 1 μg ml^{-1} and 4 °C for 1 h. Then, protein G- and A-Sepharose was added and the

reaction mixture was incubated at 4 °C for overnight. The beads were washed four times and immunoprecipitated proteins were separated using SDS-PAGE. Proteins were transferred to a nitrocellulose membrane which was subjected to immunoanalysis and resultant bands were visualized using ECL.

GST-pulldown assay. GST fusion proteins were expressed in *Escherichia coli* and purified using glutathione–Sepharose 4B beads (Amersham Pharmacia). A 1 μg sample of purified protein was incubated with protein labelled during *in vitro* translation [using the T7-coupled Transcription/Translation system (Promega)] in binding buffer [20 mM HEPES (pH 7·4), 100 mM NaCl, 0·5 % NP-40 and protease inhibitors]. Glutathione–Sepharose 4B beads were then added and the reaction mixture was incubated at 4 °C overnight. Beads were washed four times with binding buffer and bound proteins were separated by SDS-PAGE. Radioactivity was measured by autoradiography or by using a Fujix BAS-1500 screen (Fuji).

β-Gal assay of K8 transactivation function using yeast SNF5Δ. The two yeast strains FY22 (MAT**a** *his3Δ200 ura3-52*) and FY1658 (MAT**a** *his3Δ200 ura3-52 lys2-128δ snf5Δ2*) were gifts from F. Winston (Harvard Medical School, MA, USA) and were described previously (Lee *et al.*, 2002). Media was prepared according to standard methods. Yeast strain FY22 and its isogenic mutant strain SNF5Δ (FY1658) were transformed using the lithium acetate polyethylene glycol method. β-Galactosidase activity was determined in triplicate from pools of three independently transformed colonies.

RESULTS

K8 is a weak transactivator but its deletion mutant K8(1–115) has strong activity

To identify cellular proteins that interact with K8, we aimed to screen a B cell cDNA library using the *Saccharomyces cerevisiae* two-hybrid system (Guarante, 1983). Because proteins with intrinsic transactivating properties may cause false results, we checked for autonomous activation of K8 before using a DBD-fused K8 plasmid in library screening. To test the intrinsic transactivating properties of K8, we constructed a plasmid vector, pLexA/K8, which expressed a fusion protein with the DBD of the LexA protein linked to the K8 protein. When Leu^- auxotrophic yeast EGY48 transformants harbouring plasmids p8op-lacZ (*lacZ* reporter gene, URA3 selection marker in yeast) and pLexA/K8 (HIS3 selection marker in yeast) were grown on plates containing His^-, Leu^- and Ura^- minimal synthetic drop-out medium (SD/−His/−Leu/−Ura), we observed colonies on Leu^- medium. This indicates that pLexA/K8 has intrinsic transactivating properties because the integrated LEU2 nutritional reporter gene in the host cell EGY48 is under absolute control of LexA operators.

To investigate the transcriptional activation domain of K8, we constructed a set of plasmid vectors, the pLexA/K8 series (Fig. 1A). When EGY48 transformants harbouring p8op-lacZ plus members of the pLexA/K8 series were grown on synthetic medium (−His/−Leu/−Ura), we observed that only some grew on Leu^- medium. The same results were obtained using synthetic minimum medium (−His/−Ura) with X-Gal, in which β-galactosidase expression from the p8op-lacZ plasmid under the control of LexA operators was indicated by blue-coloured colonies (data not shown).

Analysis of the data obtained using various mutants showed that wild-type K8 had weak transactivating properties, as indicated by light blue-coloured colonies on synthetic minimum medium (−His/−Ura) with X-Gal plates, while K8(1–158) and K8(1–75) provided weaker and no transcriptional activation, respectively. K8(1–115) had the strongest transactivating activity, as indicated by the dark blue colonies (data not shown).

To determine whether similar data would be obtained in a mammalian system, we constructed chimeric protein expression vectors in which Gal4–DBD was fused to K8 deletion mutants (the pM/K8 series). Hybrid proteins were assayed for their ability to transactivate the Gal4-dependent promoter (pFR-luc). We found that, as in yeast, K8(1–115) had much stronger transcriptional potential than wild-type K8 (Fig. 1B). Next we subcloned the same set of deletion mutants into the pcDNA3-Gal4 vector to determine protein expression levels, since the pM vector is not suitable for such analysis in our system. Similar results as those described above were obtained when these pcDNA3-Gal4 K8 constructs were tested in reporter assays (data not shown), and immunoblotting of each construct showed that the differences in transactivating ability between K8 constructs was not due to differences in protein expression (Fig. 1C). Although there are no published data regarding K8 transactivating properties, or K8 response elements in promoters, our data suggest K8 may have domains for both transcriptional activation and suppression within K8 itself and acts as a strong transactivator under specific conditions.

K8 interacts with hSNF5 in yeast

The experiments above identified K8 deletion mutants from both the amino- and carboxyl-terminal ends that do not have transactivating properties in yeast, namely K8(1–75) and K8(116–237). We used K8(1–75) in yeast two-hybrid screening since the characteristics of the amino-terminal region of K8 are relatively unknown.

To investigate cellular proteins that interact with K8(1–75), we performed a yeast two-hybrid assay using a B cell cDNA library. The library was cloned into the pB42AD vector (TRP1 selection marker in yeast), which contains a transcriptional activation domain and expression of activation domain-fused proteins from this vector is controlled by the Gal1 inducible promoter. About 3×10^5 colonies were screened in the two-hybrid assay and 37 positive clones were identified and sequenced. Five of the 37 clones contained cDNAs that encoded a truncated amino-terminal version of the hSNF5 protein, a component of the SWI–SNF complex (Kalpana *et al.*, 1994). hSNF5 is the human homologue of the *S. cerevisiae* SNF5 protein (ySNF5) (Laurent *et al.*, 1990) and the proteins share 33–55 % overall sequence identity and 41–71 % similarity in conserved residues (Kalpana *et al.*, 1994).

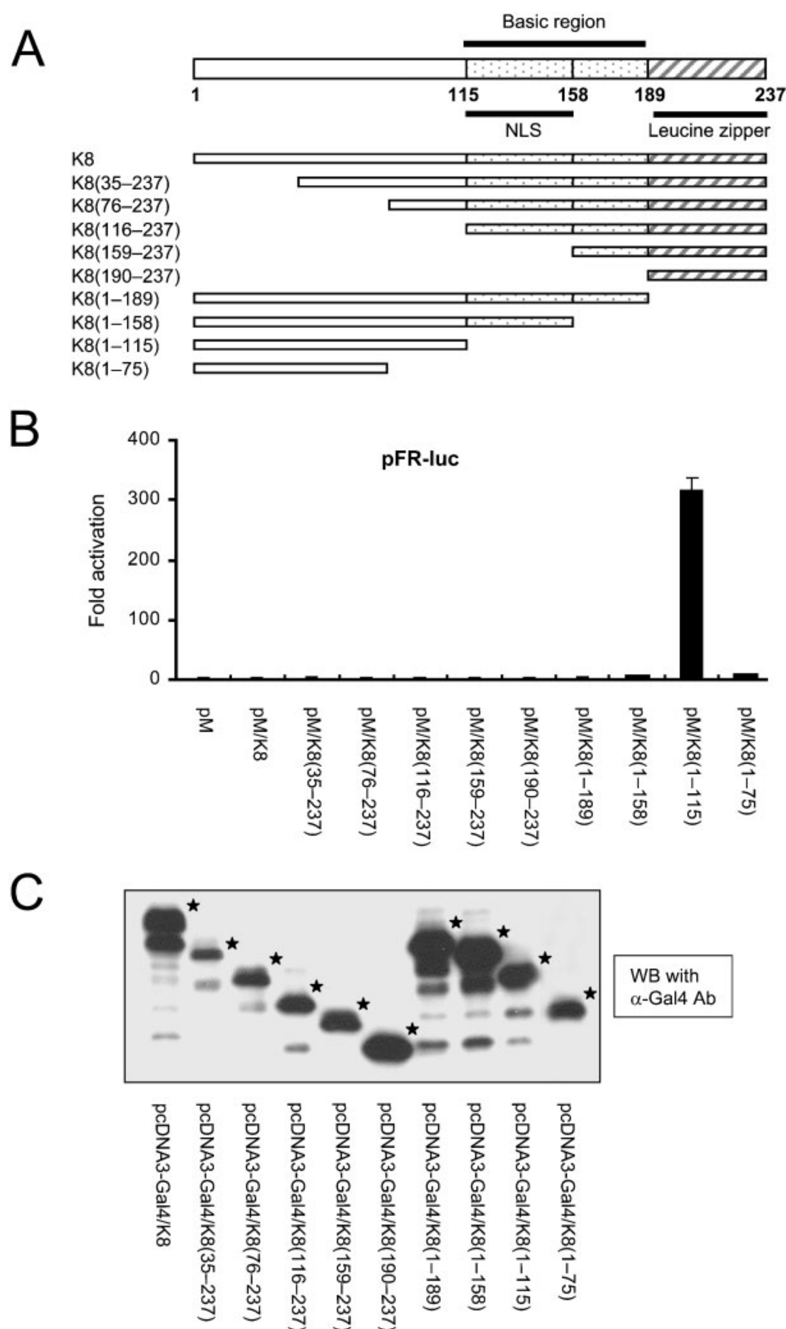


Fig. 1. K8 has weak transactivating property but its deletion mutant K8(1–115) has strong activity. (A) Schematic representation of K8 and its deletion mutants. (B) Fold activation of Gal4-dependent promoter activity (pFR-luc) for each Gal4–DBD-fused K8 deletion mutant (the pM/K8 series). Results are representative of three independent experiments. (C) Expression level of each Gal4–DBD-fused K8 series protein, as determined by immunoblotting against Gal4–DBD. WB, Western blot; Ab, antibody.

K8 co-immunoprecipitates with hSNF5 in 293T cells

To test whether KSHV K8 interacts with hSNF5 in mammalian cells, 293T cells were transfected with no DNA (mock), GST-fused K8 expression plasmid (pEBG/K8) alone, Flag-tagged hSNF5 expression vector (pME18S/hSNF5) plus blank vector (pEBG) or pME18S/hSNF5 plus pEBG/K8. We found that addition of glutathione–Sepharose beads to lysates from cells transfected with pME18S/hSNF5 plus pEBG/K8 resulted in precipitation of GST-fused K8 with Flag–hSNF5 (Fig. 2A). This was not observed when using extracts from cells transfected with pME18S/hSNF5 plus blank vector. An antibody against Flag also co-precipitated Flag-tagged hSNF5 and GST-K8. These data suggest K8 and hSNF5 are associated in mammalian cells.

K8 binds to hSNF5 in BCBL-1 cells

To investigate possible interactions between endogenous K8 and hSNF5, we performed co-immunoprecipitation assays using the KSHV-positive BCBL-1 cell line. BCBL-1 cells, with or without TPA induction (48 h), were harvested and the lysates were incubated with either rabbit polyclonal anti-HA antibody (α-HA) or rabbit hSNF5-specific polyclonal antibody (α-hSNF5). We found that K8 co-immunoprecipitated with hSNF5 from TPA-induced cells but not from uninduced cells (Fig. 2B). K8 did not

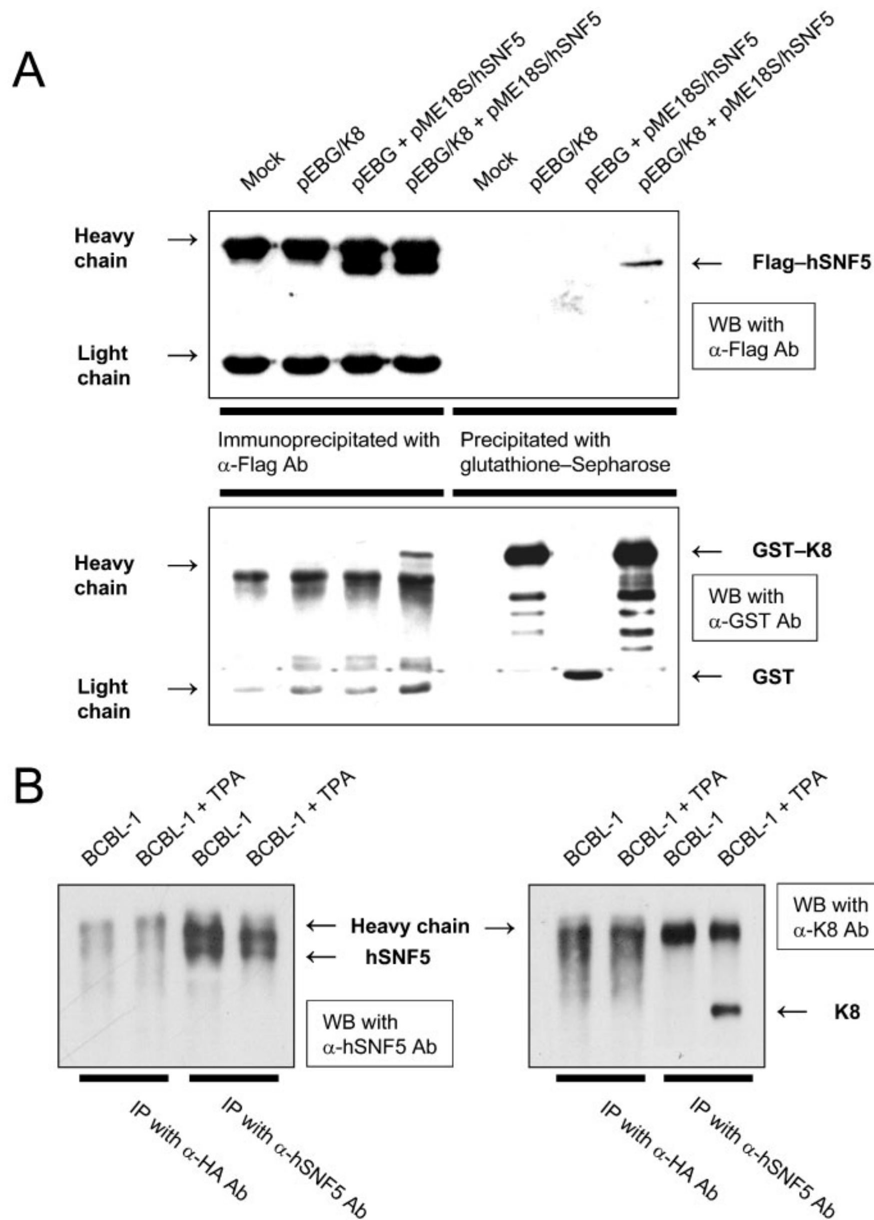


Fig. 2. K8 interacts with hSNF5 in mammalian cells. (A) 293T cells were transfected with the indicated plasmids, proteins were precipitated with α-Flag or glutathione–Sepharose from cell lysates and analysed by immunoblotting with α-Flag or α-GST. (B) Co-immunoprecipitation assays using the KSHV-positive BCBL-1 cell line. BCBL-1 cells, with or without TPA induction (48 h), were harvested and the lysates were precipitated with either α-HA or α-hSNF5 and analysed by immunoblotting with α-K8 and α-hSNF5. WB, Western blot; IP, immunoprecipitation; Ab, antibody.

co-immunoprecipitate with HA. These results show that endogenous K8 in KSHV-positive cells binds hSNF5.

K8 binds to aa 48–183 of hSNF5

To determine the K8-binding domain in hSNF5, we performed binding studies, GST-pulldown assays, using *in vitro*-translated K8 and hSNF5-deletion mutants fused to GST (Fig. 3A). hSNF5 has three highly conserved regions (CR), two of which are imperfect repeats (CR I and CR II). The integrase (IN) of HIV and c-Myc specifically binds to CR I (Cheng *et al.*, 1999; Kalpana *et al.*, 1994; Yung *et al.*, 2001). Wild-type and mutant GST-fused hSNF5 were expressed, purified and incubated with *in vitro*-transcribed and -translated ^{35}S-labelled K8. We found that K8 bound to GST–hSNF5 but not to GST protein alone (Fig. 3B). K8 bound to the amino-terminal end of hSNF5 (aa 1–183), particularly to the regions of aa 48–100 and 101–183 but did not bind to the carboxyl-terminal end (aa 184–385) of hSNF5. These data are consistent with those obtained from

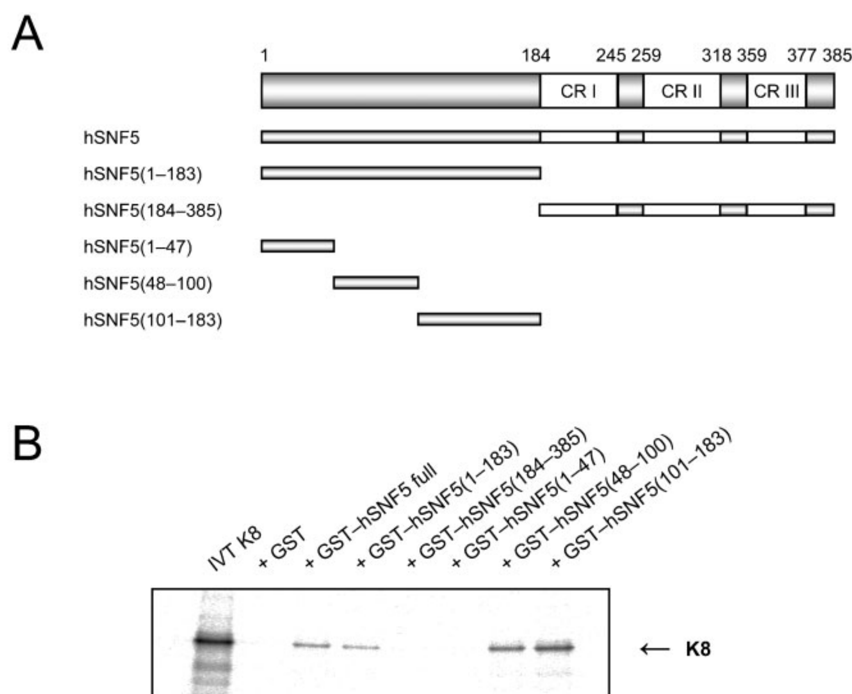


Fig. 3. Identification of the hSNF5 domain that interacts with K8. (A) Schematic representation of hSNF5 and its deletion mutants used in GST-pulldown assays. CR, Conserved region. (B) GST-pulldown assays with *in vitro*-translated, ^{35}S-labelled K8 and GST-fused hSNF5 fragments. The ^{35}S-labelled K8 proteins were incubated with 1 μg GST-fused hSNF5 fragments and the reaction mixture was precipitated by glutathione–Sepharose 4B. Bound proteins were analysed by SDS-PAGE and visualized by autoradiography.

the yeast two-hybrid assay. This is the first report of a protein interacting with this portion of hSNF5.

hSNF5 binds to aa 1–75 of K8

In vitro GST-pulldown assays were not successful in determining the hSNF5-binding domain in K8 (data not shown), therefore *in vivo* assays were performed. hSNF5 or K8 and its deletion mutants (Fig. 4A) were subcloned into the GST-fusing vector pEBG or into the Flag-tagging vector pME18S. Plasmids were transfected into 293T cells and cell extracts prepared. We found that glutathione–Sepharose precipitated GST–K8, GST–K8(1–115) and GST–K8(116–237) and it also co-precipitated Flag–hSNF5 from the cell extract that was co-transfected with K8 and K8(1–115) (Fig. 4B). This did not occur when using extracts of cells transfected with K8(116–237). These data are consistent with the results of the yeast two-hybrid assay showing K8(1–75) can bind hSNF5.

We characterized further the region of K8 required for binding to hSNF5 by constructing plasmids pME18S/K8(35–237), pME18S/K8(76–237) and pME18S/K8(116–237). We found that none of the K8 fragments expressed from these plasmids co-immunoprecipitated with GST–hSNF5, while full-length Flag–K8 did (Fig. 4C). These data suggest aa 1–34 of K8 are required for binding to hSNF5. We then tested whether K8(1–34) is sufficient for binding. Data presented in Fig. 4(D) show that glutathione–Sepharose beads precipitated GST–K8, GST–K8(1–34), GST–K8(1–75) and GST–K8(1–115) and that Flag–hSNF5 co-precipitated with all of these except K8(1–34). These results indicate K8(1–34) is necessary but not sufficient for binding to hSNF5 and that aa 1–75 of K8 are required for hSNF5 binding. This region of K8 contains the portion nominated as an acidic domain (12 of 42 residues between residues 6 and 47) (Zhu *et al.*, 1999).

Transactivation by K8 and K8(1–115) is affected by SNF5 in yeast

The multiprotein SWI–SNF complex functions by altering chromatin structure and is essential for transcriptional activation of several inducible genes (Peterson & Herskowitz, 1992). Genetic and structural data suggest that SWI–SNF disrupts the chromatin structure at promoters to allow transcription machinery increased access. SWI–SNF proteins mediate the interaction between gene-specific transactivators and general transcription factors to help general transcription machinery bind to chromatin (Lee *et al.*, 1999). Since we found that K8 and K8(1–115) have transactivating property in yeast, their ability to interact with modifiers of chromatin structure was tested.

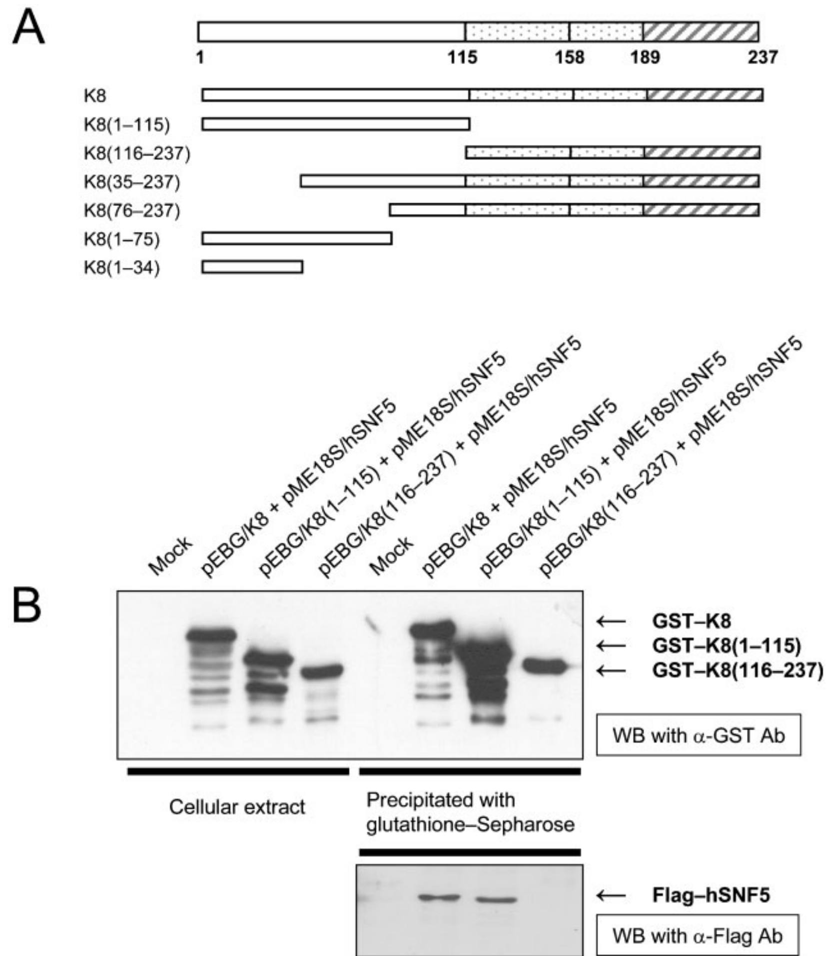


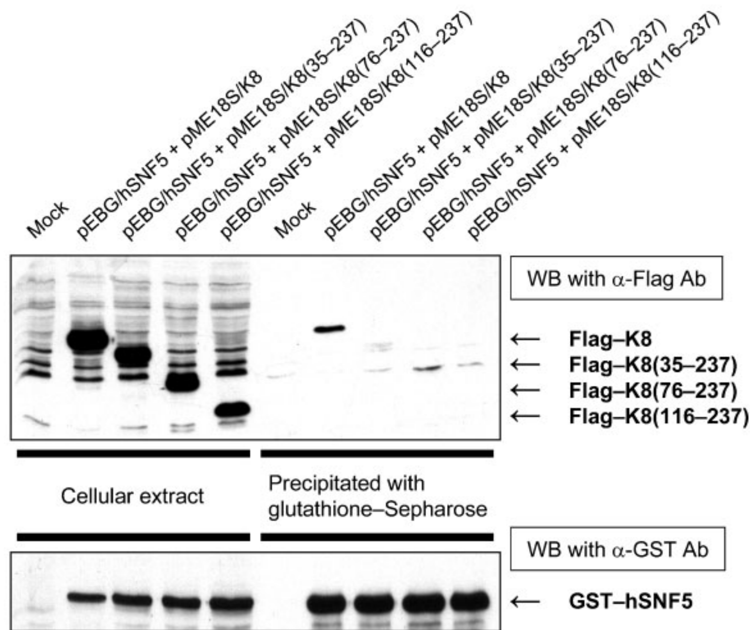
Fig. 4. For legend see page 672.

Both wild-type (FY22) and mutant yeast strains deficient in SNF5 (FY1658), the yeast homologue of hSNF5, were used in these studies. K8 and its carboxyl-terminal deletion mutant K8(1–115) were fused to the DBD of LexA and examined for activation of a *lacZ* gene in wild-type and mutant yeast, as described previously (Kowenz-Leutz & Leutz, 1999). The data presented in Table 1 and Fig. 5(A) show that LexA/K8(1–115) activation of the *lacZ* gene was greater than that by LexA/K8 and reached a level similar to activation by LexA/Gal4–DBD. In addition, these data show that *lacZ* gene activation by LexA/K8 and LexA/K8(1–115) was affected by the presence of SNF5: SNF5 enhances the transcriptional activity of LexA/K8(1–115). In accordance with published results (Laurent & Carlson, 1992), LexA alone (negative control) did not affect expression in wild-type or mutant yeast but expression driven by the LexA/Gal4 control was SNF5 dependent (Fig. 5A). Immunoblotting for LexA and β-actin expression in wild-type and mutant yeast strains confirmed that the differences in *lacZ* gene activation between constructs were not due to variations in protein levels (Fig. 5B). These data indicate that K8- and K8(1–115)-dependent gene activation in yeast requires SNF5 and probably a functional SWI–SNF complex.

DISCUSSION

Eukaryotic cell DNA is wrapped around histone octamers within the nucleus. These histone–DNA complexes form a potent obstacle for biological processes requiring access to DNA, such as transcription, replication or DNA repair. The cellular machinery allowing transcription factors to gain access to their target promoters can be divided into two categories; the histone acetyltransferases (HATs) and the chromatin-remodelling complexes. HATs add acetyl groups to the amino-terminal tails of histones and it is likely that the loss of positive charge modifies interactions between histone tails and the DNA or neighbouring nucleosomes. Chromatin-remodelling complexes have a different mechanism of operation, using the energy of ATP hydrolysis to weaken interactions between histone core particles and DNA. The SWI–SNF complex is the prototype chromatin-remodelling complex and was initially characterized in *S. cerevisiae* through genetic and biochemical studies (Kornberg & Lorch, 1999; Muchardt & Yaniv, 1999, 2001; Vignali *et al.*, 2000).

The hSNF5 (INI1 or BAF47) gene encodes a member of the SWI–SNF chromatin ATP-dependent remodelling



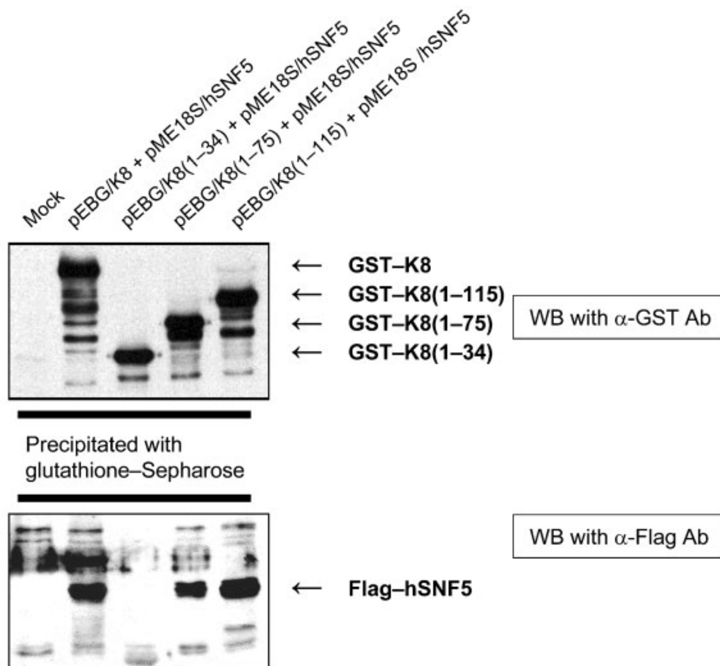


Fig. 4. hSNF5 binds to aa 1–75 of K8. (A) Schematic representation of K8 and its deletion mutants used for *in vivo* GST-pulldown assays. (B) *In vivo* GST-pulldown assays with Flag-fused hSNF5 and GST-fused K8, K8(1–115) and K8(116–237). 293T cells were transfected with the indicated plasmids and the lysates were precipitated with glutathione–Sepharose and analysed by immunoblotting with α-Flag or α-GST. (C) *In vivo* GST-pulldown assays with GST-fused hSNF5 and Flag-fused K8, K8(35–237), K8(76–237) and K8(116–237). (D) *In vivo* GST-pulldown assays with Flag-fused hSNF5 and GST-fused K8, K8(1–115), K8(1–75) and K8(1–34). WB, Western blot; Ab, antibody.

complex family and is a homologue of *S. cerevisiae* SNF5, a transcriptional activator that acts in a complex with several other proteins, including SWI2–SNF2, SNF6, SWI1 and SWI3 (the SWI–SNF complex) to activate genes (Kalpana *et al.*, 1994; Laurent & Carlson, 1992; Laurent *et al.*, 1990). hSNF5 appears to be present in all mammalian SWI–SNF complexes (Vignali *et al.*, 2000) and appears to be involved in HIV integration, association with PML and the HIV preintegration complex, interaction with the carboxyl-terminal SET domains of ALL-1 and c-Myc and EBV nuclear protein 2 transactivation (Cheng *et al.*, 1999; Kalpana *et al.*, 1994; Morozov *et al.*, 1998; Rozenblatt-Rosen *et al.*, 1998; Turelli *et al.*, 2001; Wu *et al.*, 1996, 2000; Yung *et al.*, 2001).

Recently, hSNF5 was shown to be tumour suppressor gene located on chromosome 22q11.2 and biallelic mutations in the hSNF5/INI1 gene were shown to cause malignant rhabdoid tumours, an extremely aggressive cancer of early childhood (Versteege *et al.*, 1998). Alterations in the hSNF5/

Table 1. Transactivation by K8 and K8(1–115) is affected by SNF5 in yeast

pLexA constructs†	β-Galactosidase activity*					
	Experiment 1		Experiment 2		Experiment 3	
	FY22 (wt)	FY1658 (SNF5Δ)	FY22 (wt)	FY1658 (SNF5Δ)	FY22 (wt)	FY1658 (SNF5Δ)
Control	11	11	5	4	4	3
Gal4AD	11 182	5 806	13 994	5 464	10 744	5 641
K8	65	34	46	24	8	4
K8(1–115)	7 225	1 884	7 576	1 463	9 438	3 966

*Fold activation of *lacZ* in FY22 (wt) and FY1658 (SNF5Δ) strains. Target plasmid was p8op-lacZ.
†Gal4 activation region, K8 and K8(1–115) were fused to the LexA DBD, as indicated.

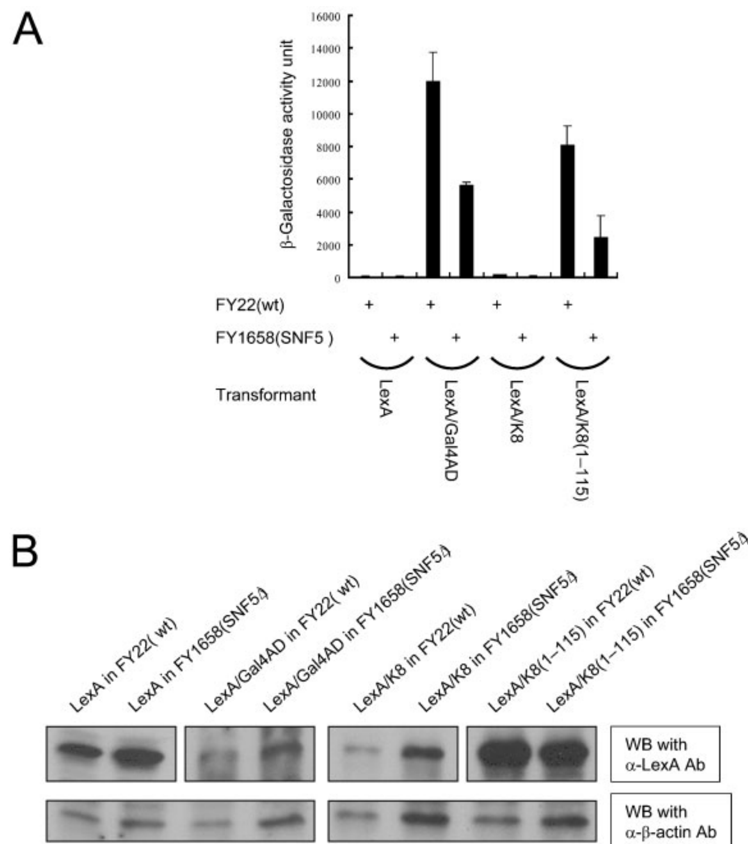


Fig. 5. Transactivation by K8 and K8(1–115) requires SNF5 in yeast. (A) β-Galactosidase activity of LexA, LexA/Gal4AD, LexA/K8 and LexA/K8(1–115) in wild-type (FY22) and SNF5-deficient (FY1658) strains. Values of β-galactosidase are given as units. Values are the average calculated from three different colonies expressing the specified fusion proteins. (B) Immunoblotting for LexA and β-actin in wild-type and SNF5-deficient strains. WB, Western blot; Ab, antibody.

INI1 gene are also found in chronic myeloid leukaemia, lymphoid malignancy and various malignancies of the CNS (Grand *et al.*, 1999; Sevenet *et al.*, 1999a, b; Yuge *et al.*, 2000). In mice, inactivation of the SNF5/INI1 gene by homologous recombination is lethal at a very early stage and heterozygous SNF5/INI1 mutants show increased susceptibility to early onset of cancer (Klochendler-Yeivin *et al.*, 2000).

In this study, we used yeast two-hybrid screening to show that K8 interacts with hSNF5. In addition, K8 was found to bind to, and co-localize with, hSNF5 in mammalian cells and to co-immunoprecipitate with hSNF5 in KSHV-harbouring BCBL-1 cells. The interaction between K8 and hSNF5 required aa 48–183 of hSNF5 and 1–75 of K8. We also found that while full-length K8 had weak transactivating properties, its carboxyl-terminal deletion mutant K8(1–115) had

strong activity. Of particular note was that the transactivating property of K8 and K8(1–115) in yeast requires SNF5, a yeast homologue of hSNF5.

Previously, we demonstrated that KSHV K8 protein interacts with CBP and that interaction might lead to competition for limited amounts of CBP (Hwang *et al.*, 2001). Because CBP has HAT activity and is used by viral proteins to promote viral gene transactivation (Arany *et al.*, 1995; Dorsman *et al.*, 1997; Eckner *et al.*, 1996; Gwack *et al.*, 2001), there is a possibility that K8 may function as a transcriptional activator of KSHV gene transcription, like its homologue EBV Zta. Recruitment of modifiers and remodellers to specific DNA sites within chromatin plays a critical role in controlling gene expression. Since K8 can interact functionally with chromatin remodellers, such as CBP and hSNF5, we can speculate that K8 modulates KSHV promoters and/or cellular promoters, by targeting chromatin-remodellers or -modifying factors.

Wu *et al.* (2001) showed the association and co-localization of K8 with the KSHV pseudoreplication compartment structure and PML in PODs. hSNF5 was also recently found to be required for efficient replication of human papillomavirus DNA and also for mammalian DNA replication, for which it acted either alone or complexed with SWI–SNF (Lee *et al.*, 1999). The data associating PML with virus replication (Adamson & Kenney, 2001; Bell *et al.*, 2000) suggest further that K8 plays a role in KSHV replication. This hypothesis is also supported by reports suggesting that phosphorylation of K8 by CDKs may link KSHV DNA replication to the cell cycle (Polson *et al.*, 2001). Further support can be inferred from data showing p53 is sequestered in virus replication centres and that K8 recruits p53 to PODs (Fortunato & Spector, 1998; Katano *et al.*, 2001).

The present work is the first to indicate that K8 may function as a transcriptional activator in the life cycle of KSHV and that there may be K8-responsive promoters. These findings are consistent with those showing K8 binds to a 300 bp sequence derived from the KSHV genome (Lin & Yuan, 2001).

Based on the present data, it may be that a complex transactivation domain is located toward the K8 amino terminus and the central region of the protein contains negative regulatory domains that can modify the transactivating function of the protein (Kowenz-Leutz *et al.*, 1994; Williams *et al.*, 1995). Such an arrangement would explain the high activity of K8(1–115) due to the lack of a negative regulatory domain (probably, aa 116–158 of K8). The observation that K8 is phosphorylated by CDKs (Polson *et al.*, 2001) suggests cell cycle-dependent changes in the phosphorylation state of K8 may modulate its function, for example, by triggering differential activation of host or viral genes at different stages. Although these possibilities need more investigation, we propose that K8 functions as a transcriptional activator under specific conditions and that it acts in concert with the cellular chromatin-remodelling factor hSNF5.

K8 binding to hSNF5 may have a number of significant cellular consequences. For example, KSHV-induced tumourigenesis may be enhanced if K8 binding interferes with hSNF5 tumour suppressor activity. In addition, K8 binding to hSNF5 may influence HIV infection, since hSNF5 is also known as HIV INI1 and is important for HIV-1 virion production (Kalpana *et al.*, 1994; Yung *et al.*, 2001). Also, K8 may function as a link between KSHV and HIV, since Kaposi's sarcoma is the most common tumour associated with HIV-1 infection (Blattner, 1999; Reitz *et al.*, 1999). Ongoing study will focus on the contribution of hSNF5 to these events.

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