Kaposi’s sarcoma-associated herpesvirus (human herpesvirus-8) encodes a homologue of the Epstein–Barr virus bZip protein EB1

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Analysis of the recently completed genomic sequence of Kaposi’s sarcoma-associated herpesvirus (human herpesvirus-8) revealed that ORF 50 encodes a protein with homology to the Epstein–Barr virus (EBV) transcription factor R. In this report, we show that ORF K8, contiguous to ORF 50, is interrupted by two introns and that the spliced RNA is translated into a bZip protein that has homology to the EBV transcription factor EB1. The newly characterized K8 protein forms homodimers but does not heterodimerize with other members of the bZip protein family.

Kaposi’s sarcoma-associated herpesvirus (KSHV) [human herpesvirus-8 (HHV8)] is a recently discovered gamma-herpesvirus classified in the genus Rhadinovirus. Initially characterized from Kaposi’s sarcoma (KS) tissue (Chang et al., 1994), it is also associated with two other neoplastic disorders: primary effusion lymphoma (PEL) [also termed ‘body cavity-based lymphoma’ (BCBL)] and multicentric Castleman’s disease (MCD) (for a review see Schulz, 1998). KSHV-infected B-cell lymphoma cell lines (often dually infected with EBV) have been established from PEL (Arvanitakis et al., 1996; Cesarsman et al., 1995; Gaidano et al., 1996; Renne et al., 1996). In these cell lines the viral transcription pattern is mainly latent, i.e. there is no production of viral particles but the productive cycle can be activated by treatment of the B cells with chemical agents such as sodium butyrate or phorbol esters (Miller et al., 1997; Moore et al., 1996; Renne et al., 1996). The recent completion of the entire KSHV genomic sequence and its analysis (Russo et al., 1996) has revealed that many open reading frames are similar to those of other rhadinoviruses, including Epstein–Barr virus (EBV) (Neipel et al., 1997). EBV is a human gamma-herpesvirus which immortalizes B cells in vitro and persists latently in these cells (for a review see Kieff, 1996). The lytic cycle can also be activated by chemical treatment with sodium butyrate or 12-O-tetradecanoylphorbol 13-acetate (TPA) (Kieff, 1996). Two immediate early EBV gene products, R (also called Rta) and EB1 (also called ZEBRA or Zta) have been shown to be required for the switch from latency to the lytic cycle (Chevallier-Greco et al., 1986; Countryman & Miller, 1985; Miller et al., 1984; Rooney et al., 1989). R and EB1 are transcriptional activators which activate expression of the EBV early genes (Buisson et al., 1989; Chavrier et al., 1989; Chevallier-Greco et al., 1989; Cox et al., 1990; Holley-Guthrie et al., 1990). Furthermore, EB1 is required for activation of the lytic origin of replication of EBV (Fixman et al., 1992; Schepers et al., 1993). Both proteins directly bind specific sites on the DNA (Flemington & Speck, 1990a; Gruffat et al., 1990, 1992; Gruffat & Sergeant, 1994; Lieberman et al., 1990). Although the R binding domain appears to be unique, the EB1 binding domain belongs to the bZip domain family (Chang et al., 1990; Farrell et al., 1989; Flemington & Speck, 1990b; Giot et al., 1991; Kouzarides et al., 1991). Comparison of the KSHV and EBV genomic sequences revealed the existence of a KSHV homologue of the R protein (ORF 50) but no putative KSHV equivalent of the EB1 protein (Russo et al., 1996). However, the very similar arrangement of open reading frames in the KSHV region containing ORFs 50 and K8 as compared to the EBV region including the R and EB1 ORFs (Fig. 1A), prompted us to analyse precisely the structure of the different mRNAs transcribed from the KSHV locus. We characterized three differentially spliced mRNAs: two contain the ORF encoding the gp35/37 protein, as reported by Raab et al. (1998) but the third encodes a new bZip protein.

We first characterized, by Northern blot analysis, the general pattern and expression kinetics of KSHV mRNAs generated from the ORF 50/K8 locus following TPA treatment. Poly(A)+ RNAs were extracted from BCBL1 cells, a PEL-derived cell line infected by KSHV alone (Renne et al., 1996), or BCBL1 cells treated with 20 ng/ml TPA for 8, 12, 24 or 48 h, using the FastTrack kit (Invitrogen) for isolation of mRNA. Northern blots were prepared as described previously (Manet et al., 1989) and hybridized with random-primed 32P-labelled...
probes: A, specific for ORF 50, or B, which covers the K8 and K8.1 ORFs (Fig. 1A). Using probe A, two major RNA species, 4.5 and 4.1 kb, respectively, were detected (Fig. 1B) as early as 8 h post-TPA treatment. Since the 4.5 kb transcript was also seen with probe B, this transcript most probably encodes the KSHV homologue of the R protein, as indicated by a vertical arrow. (B) Expression kinetics of mRNAs expressed from the ORF 50/ORF K8 region. (A) Collinearity of genetic maps of EBV and KSHV in the region of EBV containing the BZLF1 and BRLF1 ORF. ORFs are represented by grey boxes. Arrows inside the boxes indicate the orientation of each ORF. White boxes in the BZLF1 ORF indicate the position of introns. The EBV ORFs represented here have been previously characterized by cloning and characterization of full-length cDNAs (Manet et al., 1989). The representation of the KSHV ORFs is based on analysis of the genomic sequence. A single polyadenylation site is found in each represented region and is indicated by a vertical arrow. (B) Expression kinetics of mRNAs expressed from the ORF 50 and K8 regions. Two μg of poly(A)+ RNAs extracted from BCBL-1 cells untreated (lanes 1 and 7), treated with DMSO alone (lanes 2 and 8) for 48 h or treated with TPA (20 ng/ml in DMSO) for 8 (lanes 3 and 9), 12 (lanes 4 and 10), 24 (lanes 5 and 11) or 48 h (lanes 6 and 12) were analysed by Northern blotting. Probes A and B (represented in part A of the figure) specific for ORF 50 and ORF K8/K8.1 respectively were generated from the L56 lambda genomic clone (Russo et al., 1996). amplifications are indicated in Fig. 2(A). The first set of primers used (F9–B50) was designed to amplify transcripts containing the K8 ORF. The F9 primer (5′GCCAAATGCCACAGAATGAAGG3′) is situated at the beginning of the K8 ORF and the B50 primer (5′ATTTACTTATGTTTGTGCTACACTATGTAAGG3′) immediately upstream of the nearest polyadenylation signal. These two primers amplified a single DNA fragment of 1.2 kb (Fig. 2B, lane 1). The precise structure of the K8 mRNA determined by DNA sequencing of the 1.2 kb cDNA fragment is presented in Fig. 2(C). The K8 mRNA was generated from a precursor containing three introns, as indicated in the figure. It is noteworthy that the 5′ splice site of the second intron is 6 bp upstream of the K8 ORF termination codon deduced from the genomic sequence. A major consequence of this splicing event is to change the end of the genomic K8 ORF, by removing the termination codon and extending the K8 ORF to a new termination codon located within the second exon, at position 75791. Our choice of the B50 primer for the RT–PCR assumed that the polyadenylation signal used was at position 76714. In order to verify this hypothesis, we also used a 3′ primer, B60 (5′CCAGAAAAGGTGACCACACT3′), located downstream of the polyadenylation site. That no fragment was amplified by RT–PCR with the F9–B60 pair of primers (Fig. 2B, lane 2), strongly suggests that the polyadenylation signal used is indeed at position 76714. As a control, the same set of oligonucleotides was shown to efficiently amplify a 2 kb fragment from genomic DNA, indicating that the set of primers used was functional for PCR amplification (data not shown).

The second set of primers (F35–B50) was designed to amplify cDNAs synthesized from RNAs containing the K8.1 ORF, previously reported to code for the gp35/37 glycoprotein (Raab et al., 1998). As described above, we used the B50 oligonucleotide and F35 oligonucleotide (5′ATGAGTATCACCAGATGTCCACGCAC3′), which is located at the beginning of the K8.1 ORF. Three fragments (a, b and c) were amplified with these primers (Fig. 2B, lane 4) and their DNA sequence determined. Fragments b and c correspond to two differentially spliced mRNAs (Fig. 2C) and fragment a to an unspliced form of polyadenylated RNA. The detection of fragment a by RT–PCR did not seem to be due to genomic DNA contamination, as no unspliced form of the K8 mRNA was generated from a precursor containing three introns, as indicated in the figure. It is noteworthy that the 5′ splice site of the second intron is 6 bp upstream of the K8 ORF termination codon deduced from the genomic sequence. A major consequence of this splicing event is to change the end of the genomic K8 ORF, by removing the termination codon and extending the K8 ORF to a new termination codon located within the second exon, at position 75791. Our choice of the B50 primer for the RT–PCR assumed that the polyadenylation signal used was at position 76714. In order to verify this hypothesis, we also used a 3′ primer, B60 (5′CCAGAAAAGGTGACCACACT3′), located downstream of the polyadenylation site. That no fragment was amplified by RT–PCR with the F9–B60 pair of primers (Fig. 2B, lane 2), strongly suggests that the polyadenylation signal used is indeed at position 76714. As a control, the same set of oligonucleotides was shown to efficiently amplify a 2 kb fragment from genomic DNA, indicating that the set of primers used was functional for PCR amplification (data not shown).

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KSHV encodes a bZip protein

Fig. 2. Determination of the genetic content of K8 and K8.1 mRNA. (A) Schematic representation of the ORF K8 and ORF K8.1 region. The positions of the primers used for the RT–PCR amplifications is shown and their orientation indicated by the direction of the arrows. (B) RT–PCR amplifications using DNase I-treated poly(A)\(^+\) RNA extracted from TPA-induced BCBL-1 cells. The primers used for each amplification are indicated in the table. The RT–PCR reaction products were separated on a 2% agarose gel. (C) Map of the K8 and K8.1 cDNA amplified by RT–PCR. The cDNA fragments obtained in (B) were purified, cloned in plasmid pCR2.1 (Invitrogen) and sequenced. Exons are shown in open boxes and the coding part of the cDNA by the grey surrounding boxes. Numbers indicate nucleotide positions in the KSHV sequence (Russo et al., 1996).

the F9 and B35 primers in an RT–PCR amplification (Fig. 2B, lane 3). Comparison of the sizes of the K8 and K8.1 mRNAs characterized by RT–PCR with the sizes of the mRNAs characterized by Northern blot analysis identifies the K8 mRNA as the 1–2 kb transcript and the K8.1b mRNA as the 0–85 kb species. This was confirmed by hybridization with a probe located in the second exon of the K8.1b mRNA (data not shown). Furthermore, the timing of expression of the K8.1b mRNA correlates with the fact that the protein product (the gp35/37 glycoprotein) is expressed late in the lytic cycle (Raab et al., 1998). That the K8.1c mRNA species revealed by RT–PCR cannot be specifically identified on the Northern blot is probably due to its under-representation.

One of the consequences of the splicing events we have just characterized is to generate new ORFs that were not deduced from the genomic sequence. Interestingly, analysis of the amino acid sequence of the protein potentially expressed from the newly characterized K8 ORF reveals the presence of a putative bZip domain at its C terminus. The amino acid sequence of this bZip domain can be successfully aligned to the amino acid sequence of the bZip domains of the EB1 and c-Jun proteins (Fig. 3A). In order to determine whether the K8 protein can form homodimers, as expected from the presence of the bZip domain, we performed in vitro co-immunoprecipitation assays using the wild-type K8 protein and a truncated form. For these experiments, we generated expression plasmids for the wild-type K8 protein, a C-terminal deletion mutant lacking the bZip domain [K8 (Δ190–237)] and an N-terminal deletion mutant tagged with the Flag peptide (IBI Flag system, Kodak) [FlagK8 (Δ1–96)]. We also used expression plasmids for the EB1 protein tagged with the Flag peptide (FlagEB1), and a truncated form of EB1 [EB1 (Δ1–86)] (Giot et al., 1991). 35S-labelled proteins were expressed from these plasmids using the TnT coupled reticulocyte lysate system (Promega). A schematic representation of the different proteins is shown in Fig. 3(B). All the proteins were produced in comparable amounts (data not shown). The in vitro-translated proteins, expressed either alone or as a mixture as indicated in the upper part of Fig. 3(C), were then immunoprecipitated with monoclonal antibody (MAb) M2 directed against the Flag epitope as described (Waltzer et al., 1994) and analysed by SDS–PAGE. As shown in Fig. 3(C), M2 immunoprecipitated both FlagK8 (Δ1–96) (lanes 3 and 8) and FlagEB1 (lane 1), but not K8 (lane 4), K8 (Δ190–237) (lane 9) or EB1 (Δ1–86) (lane 2) alone. However, when K8 and FlagK8 (Δ1–96) were co-translated, K8 was co-immunoprecipitated together with FlagK8 (Δ1–96) (lane 7) by the M2 MAb, whereas when K8 (Δ190–237) was co-expressed with FlagK8 (Δ1–96) no co-immunoprecipitation could be detected (lane 11). These results strongly suggest that K8 can form homodimers and that the leucine-zipper domain is necessary for homodimerization. We then investigated whether K8 heterodimerizes with other members of the bZip family and in particular EB1. As expected, when EB1 (Δ1–86) and FlagEB1
were co-expressed. EB1(Δ1–86) could be co-immunoprecipitated together with FlagEB1 by the M2 MAb. However, when EB1(Δ1–86) was co-expressed with FlagK8(Δ1–96), no co-immunoprecipitation was observed. Thus, K8 and EB1 appear not to heterodimerize. We also tested the ability of K8 to heterodimerize with c-Jun, Fos, GCN4 and C/EBP. None of these proteins formed heterodimers with K8 in our assay conditions (data not shown). Thus K8, in a manner similar to EB1 and GCN4, seems only to form homodimers.

In conclusion, we have identified a structural homologue of the EBV EB1 protein in KSHV. Comparison of the amino acid sequence of the two proteins gives an overall identity of 18%, but more importantly we have shown that K8, like EB1, is a bZip protein. K8 is thus likely to be a DNA-binding protein whose binding specificity remains to be determined. A potential role of K8 in transcriptional activation of the KSHV lytic cycle is, however, open to question, given the results recently published by Sun et al. (1998) which show that K8 does not activate the expression of at least two lytic cycle transcripts (the abundant PAN RNA and the v-IL6 mRNA) in BC-1 cells. Nevertheless, they have not shown that K8 was expressed from their construction. In addition, genes other than the PAN and v-IL6 could be activated by K8. Hence, whether K8 is a functional homologue of EB1 in the transcriptional activation of KSHV genes or in the activation of the lytic origins of replication remains to be determined.

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Fig. 3. K8 forms stable homodimers in vitro. (A) Alignment of the bZip K8 domain with those of EB1 and c-Jun. Positions of hydrophobic amino acids involved in the coil-coiled interaction are indicated by the small open boxes and the basic amino acids present in the basic domain by black circles. Amino acid identities are shown by grey boxes. (B) Schematic representation of the different proteins used in the co-immunoprecipitation assays. The open boxes signal the position of the Flag epitope. (C) Homodimerization of K8 in vitro. The 35S-labelled in vitro-translated proteins used in the (co)immunoprecipitations are indicated in the tables. The immunoprecipitated proteins were analysed by 10% SDS–PAGE and visualized by autoradiography.

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