VIROLOGY 249, 140–149 (1998)
ARTICLE NO. VY989316



# Human Herpesvirus-8 ORF K8.1 Gene Encodes Immunogenic Glycoproteins Generated by Spliced Transcripts

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Received March 25, 1998; returned to author for revision June 8, 1998; accepted June 26, 1998

A cDNA library from phorbol ester-induced human herpesvirus-8 (HHV-8) carrying BCBL-1 cells was screened with an HIV+KS+ serum, and several cDNA clones encoding HHV-8 proteins were identified. Sequence analysis of two full-length cDNA clones show open reading frames (ORFs) encoded by spliced messages originating from the HHV-8 K8.1 gene. One cDNA encodes an ORF of 228 amino acids, designated K8.1A, with a cleavable signal sequence, a transmembrane domain, and four *N*-glycosylation sites. The splicing event generated the transmembrane domain in the ORF not seen in the genomic K8.1 ORF. Another cDNA encodes an ORF of 167 amino acids, designated K8.1B, that shares similar amino and carboxyl termini with ORF K8.1A but with an in-frame deletion. The primary translation products of ORF K8.1A (34 kDa) and K8.1B (20 kDa) in the *in vitro*–transcription–translation experiments shifted into glycosylated species of 43 and 32 kDa, respectively, in the presence of microsomal membranes. This suggested that the ORF K8.1A and K8.1B encode for glycoproteins. Riboprobes from the K8.1A cDNA insert hybridized with an HHV-8-specific 0.9-kb abundant transcript from BCBL-1 cells. Synthesis of this RNA was eliminated in the presence of a DNA synthesis inhibitor, suggesting that this RNA was a late gene transcript. Because ORFs K8.1A and K8.1B are unique for HHV-8, human sera were tested in Western blot reactions for antibodies against glutathione-*S*-transferase–ORF K8.1A fusion protein. All sera that were positive for HHV-8 antibodies in immunofluorescence assays with phorbol ester-induced BCBL-1 cells were also positive for anti-ORF K8.1A antibodies. This suggests that measurement of anti-ORF K8.1A antibodies would provide an HHV-8-specific serological assay. Further work is needed to define the biological role of the HHV-8 ORF K8.1A and K8.1B glycoproteins. 

## INTRODUCTION

Human herpesvirus-8 (HHV-8), or Kaposi's sarcoma (KS)-associated herpesvirus (KSHV), is a newly identified lymphotropic herpesvirus with a strong association with KS (Chang *et al*., 1994, 1995). HHV-8 DNA has been detected in >95% of KS lesions studied, in both HIV− and HIV+ individuals and in all clinical subtypes of KS (Ambroziak *et al*., 1995; Chang *et al*., 1994, 1996; Moore *et al*., 1995; Smith *et al*., 1997). In addition, HHV-8 sequences have been detected in body cavity-based B-cell lymphomas (BCBLs). Cell lines (BC-1, HBL-6, and BCBL-1) have been established from the BCBL tumors (Cesarman *et al*., 1995; Gao *et al*., 1996, 1996; Moore *et al*., 1996; Renne *et al*., 1996). BC-1 and HBL-6 carry both HHV-8 and human Epstein–Barr virus (EBV). BCBL-1 cells carry only HHV-8, and lytic cycles can be induced by phorbol ester (TPA) (Chan *et al*., 1998; Chandran *et al*., 1998; Renne *et al*., 1996; Smith *et al*., 1997). BCBL cells have been used in immunofluorescence and Western blot assays to measure antibody responses in human sera (Chandran *et al*., 1998; Gao *et al*., 1996a, 1996b; Kedes *et al*., 1996; Lennette *et al*., 1996; Smith *et al*., 1997).

Sequence of ~140-kb HHV-8 DNA encoding 80 complete ORFs was published very recently (Neipel *et al*., 1997; Russo *et al*., 1996). Phylogenetic analysis of the HHV-8 genome, the gene arrangements, and protein sequences deduced from these sequences show striking similarity to herpesvirus Saimiri (HVS), a Simian herpesvirus, and more distantly related to EBV; both viruses are in the γ-herpesvirus group (Albrecht *et al*., 1992; Kieff *et al*., 1996; Russo *et al*., 1996; Neipel *et al.,* 1997). These HHV-8 ORFs are currently identified as ORFs 1–75 by their similarity to HVS ORFs. Russo *et al.* (1996) also identified 15 ORFs unique for HHV-8 designated K1–K15. Neipel *et al.* (1997) identified four additional HHV-8 unique ORFs and assigned decimal K numbers for them (K4.1, K4.2, K8.1, and K10.1). Sera from HIV+KS+ and HIV+KS− individuals immunoprecipitated several HHV-8-specific polypeptides and glycoproteins (Chandran *et al.,* 1998). Characterizations of HHV-8 proteins are fundamental to a rational understanding of the biology of HHV-8 and its role in KS and other human diseases and for diagnostic purposes. As an initial step, we screened a cDNA library from TPA-induced BCBL-1 cells with an HIV+KS+ serum and identified cDNAs encoding several HHV-8 proteins (Chandran *et al.,* 1998). Among these, we

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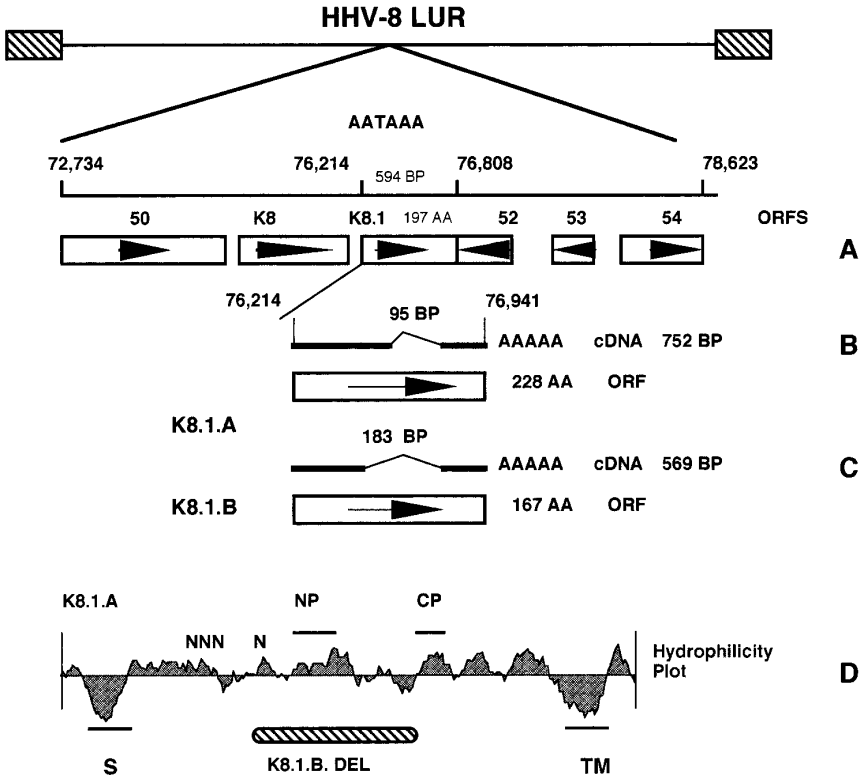


FIG. 1 Schematic representation of the cDNAs encoding the HHV-8 ORF K8.1A and K8.1B. (Top line) Schematic representation of HHV-8 long unique region (LUR). (A) schematic representation of HHV-8 ORFs. (B and C) Location of the cDNAs in the HHV-8 genome. (Numbers) Genomic nucleotide positions of ORFs (Russo *et al*., 1996). (Arrows) Direction of transcription. The cDNAs encoding ORFs K8.1A and K8.1B of 228 and 167 AA, respectively, are shown. The splice sites are shown by the inverted V, and AAAA represents the poly(A)$^+$ tail. (D) Schematic hydropathic analysis of the deduced ORF K8.1A protein sequence from the cDNA. (Peaks above the horizontal axis) Hydrophilicity. (Peaks below the horizontal axis) Hydrophobicity. (S) Putative signal sequence. (N) Putative *N*-glycosylation sites (N-X-T/S). (TM) Predicted transmembrane AA sequences. (K8.1B.DEL) AA sequences deleted in the ORF K8.1B by splicing.

have identified two cDNAs encoded by the genomic HHV-8 ORF K8.1. These two cDNAs are generated by spliced transcripts and encode immunogenic glycoproteins.

## RESULTS

### Identification of cDNA encoding glycoproteins HHV-8 ORF K8.1A and K8.1B

To identify the genes encoding immunogenic HHV-8 proteins, serum from an HIV+KS+ individual with an immunofluorescence assay (IFA) titer of 1:10,240 with induced BCBL-1 cells was used to screen the cDNA library from TPA-induced BCBL-1 cells. After four screenings, 56 cDNA clones were isolated and released in the phagemid forms by cocultivation with the helper phage. These cDNAs were sequenced from both orientations. Sequences were analyzed for ORF and compared with the published genomic HHV-8 sequence (Russo *et al.,* 1996; Neipel *et al.,* 1997). Several ORFs were identified (Chandran *et al.,* 1998). Complete HHV-8 ORF 65 was encoded in 11 cDNA clones. In the other cDNA clones, the length of ORFs ranged from 280 to 800 amino acids (AA). Because these clones were identified by the HIV+KS+ serum, this suggests that they encode immunogenic protein regions.

Initial comparison of the nucleotide sequences of four cDNA clones from the above screening showed 100% identity to a 1200-bp region in the HHV-8 genomic sequence characterized by Russo *et al.* (1996) and Neipel *et al.* (1997) (Fig. 1A). In this region, there is an ORF designated K8.1, which starts at the genomic nucleotide position 76,214 bp, ends at 76,808 bp, and thus encodes a 197-AA-long ORF with a predicted molecular mass of ~22 kDa (Fig. 1A) (Neipel *et al.,* 1997). The genomic K8.1 ORF has an amino-terminal signal sequence and five putative *N*-glycosylation sites but no transmembrane sequence. Among the four cDNA clones identified, two cDNA clones encoded two full-length ORFs of 228 and 167 AA (Figs. 1B, 1C, and 2). A comparison of these two ORFs sequences with the genomic sequence showed that they were encoded by spliced transcripts. The larger cDNA is 752 bp long, starts at the genomic nucleotide position 76,214 bp, ends at position 76,941 bp, and uses the polyadenylation signal sequence (AATAAA) at position 77,013 bp. This cDNA encodes a 228-AA-long ORF

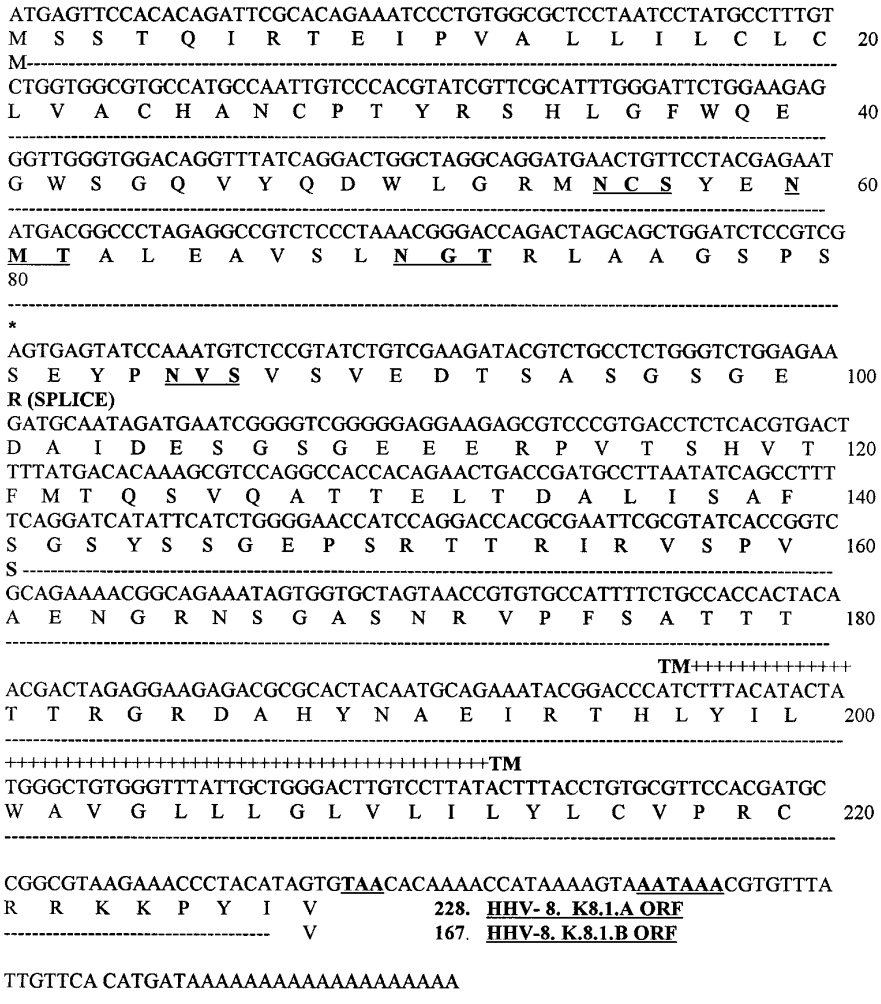


FIG. 2. Sequence analysis of the HHV-8 ORF K8.1A and K8.1B cDNA. Nucleotide and the predicted AA sequence of the full-length cDNA encoding ORF K8.1A and K8.1B are shown. The AA position is numbered on the right. The ORF termination codon (TAA), the putative polyadenylation signal sequence (ATTAAA), and putative *N*-glycosylation sites (N-X-T/S) are underlined and in bold. (+) Predicted transmembrane (TM) AA sequence. (Dotted lines) AA sequences of ORF K8.1B shared with ORF K8.1A. (*) Splice site in ORF K8.1B.

with a predicted molecular mass of ~25 kDa and is designated K8.1A. The first 142 AA encoded by the K8.1A cDNA are identical to the genomic K8.1 ORF sequence. Analysis suggested that the protein encoded by the K8.1A cDNA is a typical class I glycoprotein with a cleavable amino-terminal signal sequence or sequences, a transmembrane domain (197–213 AA), seven cysteine residues, and four putative *N*-glycosylation sites (N) (Figs. 1B, 1D, and 2). This cDNA is derived from a transcript with a 95-bp sequence spliced out [CAG/(GT)GTAT donor site and TCTAC(AG)/G acceptor site] and ends at the genomic nucleotide position 76,941 bp, which is 187 bp beyond the end of genomic ORF K8.1 (Fig. 1B). This has resulted in the generation of a transmembrane domain not seen in the genomic ORF K8.1.

The smaller cDNA is 569 bp long with a 183-bp sequence spliced out. The splice acceptor site for the ORF K8.1B transcript is the same as that for the ORF K8.1A transcript (Figs. 1C and 2). However, the splice donor site [CGA/(GT)GAGT] for the K8.1B cDNA is upstream of the splice donor site of the K8.1A cDNA, resulting in an in-frame deletion of 61 AA in the smaller ORF (Figs. 1D and 2). The resulting 167-AA-long ORF is predicted to code for a protein of ~18.5 kDa and is designated K8.1B. ORF K8.1B is also a typical class I glycoprotein with a cleavable signal sequence, a transmembrane domain, and three putative *N*-glycosylation sites (N). Except one AA change near the splice site (S to R), the ORF K8.1B shares identical AA sequences with ORF K8.1A (Figs. 1C and 2). To confirm the sequence and the splicing patterns, 5′-rapid amplification of cDNA ends (RACE) was done. RNA extracted from TPA-induced BCBL-1 cells (48 h p.i.) was used for this. Single-stranded cDNAs from TPA-induced cells were synthesized using oligo(dT) primer, and the second strand was subsequently synthesized. Double-stranded adapters were ligated to both ends of the cDNA molecule, and polymerase chain reaction (PCR) was performed using an adapter-specific

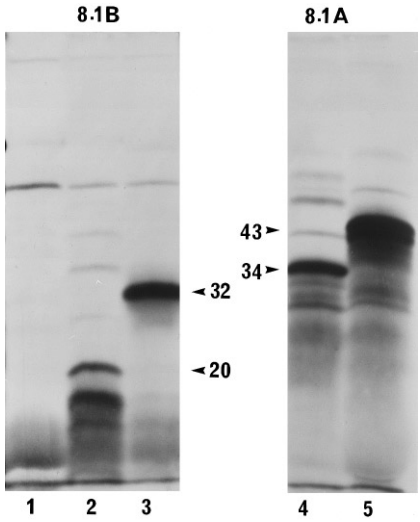


FIG. 3. *In vitro* translation of RNAs transcribed from the cDNA inserts encoding HHV-8 ORF K8.1A and K8.1B. Antisense transcripts prepared *in vitro* from the cDNA insert were translated *in vitro* using rabbit reticulocyte lysates and [$^{35}$S]methionine. (Lane 1) Translation without the addition of RNA. (Lane 2) Translation of K8.1B RNA transcribed from SP6 promoter in the absence of microsomal membranes. (Lane 3) Translation of K8.1B RNA in the presence of microsomal membranes. (Lane 4) Translation of K8.1A RNA transcribed from SP6 promoter in the absence of microsomal membranes. (Lane 5) Translation of K8.1A RNA in the presence of microsomal membranes. (Numbers) Approximate molecular mass (in kDa) of the major *in vitro* translated polypeptides and the glycosylated species. Samples were analyzed on 12% acrylamide cross-linked with *N*,*N*′-diallyltartardiamide.

primer and the ORF K8.1A- and K8.1B-specific primer corresponding to the 3′-end stop site. Several clones were cloned and sequenced. The sequences and splicing patterns were identical to those of the ORF K8.1A and K8.1B cDNAs, confirming the sequences and the splicing pattens seen in the K8.1A and K8.1B cDNAs.

## The proteins encoded by cDNAs HHV-8 ORFs K8.1A and K8.1B are glycoproteins

By AA sequence analysis, the ORFs K8.1A and K8.1B identified here are predicted to encode for glycoproteins. To verify this, *in vitro* transcription-translation experiments were done. No specific protein was detected in control reactions without the addition of any RNA (Fig. 3, lane 1). Translation of the RNA transcribed from K8.1B cDNA produced a polypeptide of ~20 kDa (Fig. 3, lane 2). With the addition of canine microsomal membranes to the translation reaction mixture, this polypeptide shifted in mobility to a polypeptide of ~32 kDa (Fig. 3, lane 3). The major primary translation product of K8.1A cDNA is a 34-kDa polypeptide (Fig. 3, lane 4), and in the presence of microsomal membranes, it shifted into a glycosylated species of 43 kDa (Fig. 3, lane 5). Treatment of these *in vitro* synthesized products in the presence of membranes with *N*-glyconase decreased the 43- and 32-kDa polypeptides into smaller polypeptides of ~34 and ~20 kDa (data not shown), indicating the removal of *N*-linked oligosaccharides. These results showed that the HHV-8 ORF K8.1A and K8.1B cDNAs encode glycoproteins.

## HHV-8 ORF K8.1A and K8.1B cDNAs are generated from a late class transcript

To define further the viral specificity of the HHV-8 ORF K8.1A and K8.1B cDNA inserts, RNA was isolated from uninduced and TPA-induced BCBL-1 and BJAB cells. Total RNAs were analyzed by Northern blot hybridization with a 0.7-kb antisense RNA probe transcribed from the HHV-8 ORF K8.1A cDNA insert. No hybridization was detected with total RNAs from uninduced and TPA-induced BJAB (HHV-8−) cells (Fig. 4A, lanes 1 and 2). Under the conditions of high stringency, the K8.1A RNA probe hybridized specifically to a prominent band of ~0.9 kb and a less prominent band of ~0.7 kb from uninduced and TPA-induced cells (48 h p.i.) (Fig. 4A, lanes 3 and 4). Longer exposure of the autoradiograph was required to detect the hybridization with uninduced BCBL cell RNA. Longer exposure also revealed several additional RNAs from uninduced and TPA-induced BCBL-1 cells (Figs. 4A, lanes 3 and 4, and 4B, lanes 3–7),

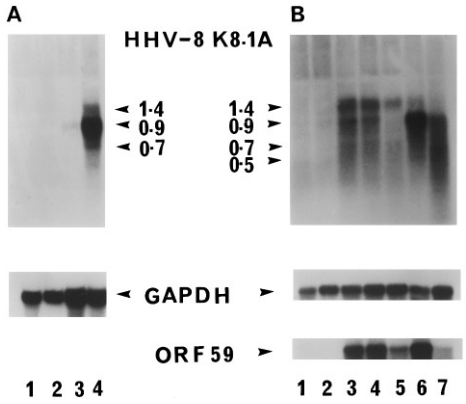


FIG. 4. (A, Top) Identification of transcripts hybridized with the riboprobe from the ORF K8.1A cDNA insert. Total unfractionated RNA was isolated from uninduced and TPA-induced BJAB (lanes 1 and 2) and from uninduced and TPA-induced BCBL-1 cells (lanes 3 and 4). Samples were analyzed by Northern blot hybridization with a radiolabeled, *in vitro* transcribed RNA probe synthesized from the 0.7-kb ORF K8.1A cDNA insert. (A, Bottom) Membrane from A was stripped and probed with a radiolabeled GAPDH RNA probe. The sizes (in kb) of the hybridized RNAs are indicated. Standard RNA marker molecules of known sizes were included in parallel lanes. (B) Kinetics of RNAs identified by HHV-8 ORF K8.1A insert. (Top) Total unfractionated RNA was isolated from cells. (Lane 1) Uninduced BJAB. (Lane 2) TPA-induced BJAB cells. (Lane 3) Uninduced BCBL-1 cells. (Lane 4) BCBL-1 cells collected after 24 h of TPA induction. (Lane 5) BCBL-1 cells collected after 24 h of PAA and TPA treatment. (Lane 6) BCBL-1 cells collected after 48 h of TPA induction. (Lane 7) BCBL-1 cells collected after 72 h of TPA induction. This autoradiograph was exposed longer than the one shown in Figure 4A. (Middle) Membrane from B was stripped and probed with a radiolabeled GAPDH RNA probe. (Bottom) Membrane from the middle was stripped and probed with a riboprobe synthesized from an HHV-8 cDNA encoding the early-late HHV-8 ORF 59 protein (Chan *et al*., 1998).

with the 0.9-kb species being the most abundant (Figs. 4A and 4B). The calculated sizes of the other faint RNA bands detected were 1.4 and 0.5 kb (Figs. 4A and 4B). Because the 0.9-kb transcript was similar in size to the cDNA clones of ORF K8.1A and K8.1B, we concluded that this transcript must arise from the K8.1 gene. To confirm that an equal amount of RNA was loaded in each sample and to verify the integrity of the RNA samples, the membrane was stripped and then rehybridized with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe, a cellular constitutive enzyme expressed in all cells. No appreciable difference in the intensity of GAPDH RNA was observed in samples from uninduced and TPA-induced BCBL-1 and BJAB cells (Fig. 4A).

To determine the kinetics of the transcript identified by HHV-8 ORF K8.1A insert, RNA extracted from TPA-induced cells collected at 24, 48, and 72 h p.i. was hybridized with the antisense RNA probe transcribed from the K8.1A cDNA insert (Fig. 4B). This autoradiograph was exposed for a longer time than the autoradiograph shown in Figure 4A. No hybridization was detected with total RNA from uninduced and TPA-induced BJAB (HHV-8−) cells (Fig. 4B, lanes 1 and 2). All the different RNA species hybridized with the K8.1A probe could be detected in both uninduced and TPA-induced cells (24 h p.i.) (Fig. 4B, lanes 3 and 4). The highest level of the 0.9-kb message was detected at 48 h p.i. (Fig. 4B, lane 6) and reduced by 72 h p.i. (Fig. 4B, lane 7). In contrast, the synthesis of the 1.4-kb band decreased appreciably by 48 h and was barely detectable by 72 h. This indicated differential regulation of the transcripts from the HHV-8 ORF K8.1A gene locus.

To determine whether the HHV-8 ORF K8.1A RNA transcript belongs to the early or late class of transcripts expressed in lytic infection, TPA-induced BCBL-1 cells were incubated with PAA for 24 h. At 24 h p.i., the 0.9-kb transcript was not detectable in the TPA-induced, PAA-treated cells (Fig. 4B, lane 5). In contrast, the 1.4-kb transcript was detected in the presence of PAA but in a lower amount than the untreated control (Fig. 4B, lanes 4 and 5). When this membrane was stripped and rehybridized with the GAPDH riboprobe, no inhibition of the GAPDH RNA by PAA was observed. In contrast, the RNA transcripts detected by a riboprobe synthesized from a cDNA encoding the early-late class HHV-8 protein P50-ORF59 (Chan *et al.,* 1998) was not completely inhibited by the PAA treatment (Fig. 4B, bottom). The complete inhibition of the major 0.9-kb transcript by PAA suggested that this transcript encoded by the HHV-8 gene locus K8.1 should be classified as truly late transcript ($\gamma$-2). The 1.4-kb RNA resembles an early class of RNA. The origin of this transcript is not known at present and may arise from the adjacent ORF K8 or ORF 50 genes encoding larger proteins. Similarly, the origin of the 0.7- and 0.5-kb RNAs is not known at present. The polyadenylation signal sequence used by the K8.1A and K8.1B transcripts also can be used by other preceding genes, such as ORF K8 and ORF 50, with different splice patterns with the 5′ part of the K8.1A and K8.1B ORF without including the 3′ end of the K8.1A and K8.1B sequences. Hence, K8.1A riboprobe still could recognize, albeit weekly, other transcripts such as the 1.4-, 0.7-, and 0.5-kb transcripts. The RACE techniques used here rely on the amplification using the primer corresponding to the 3′ stop site of K8.1A and K8.1B, and hence we specifically amplified only the clones with the intact 3′ end and not the clones with sequences corresponding to the K8.1A and K8.1B 3′ part spliced out.

## Expression of ORF K8.1A and K8.1B as a glutathione-*S*-transferase (GST) fusion protein

HHV-8 ORFs K8.1A and K8.1B were subcloned into GST-expression vectors and induced to express the fusion proteins. The expected sizes of ORF K8.1A and K8.1B fusion proteins were 52–54 and 42–44 kDa, respectively. To verify their expression in bacteria, whole bacterial cell extracts were tested in Western blot reaction with rabbit anti-ORF K8.1A peptide antibodies and human sera. The rabbit antibodies, designated Ra8.1A-NP and Ra8.1A-CP, were raised against synthetic peptides corresponding to ORF K8.1A AA residues 93–111 and 140–156, respectively (Fig. 2). Because residues 93–111 are not present in the ORF K8.1B protein due to splicing, antibodies against this peptide were not expected to react with ORF K8.1B. However, residues 140–156 are common to both ORFs, and hence the Ra8.1A-CP antibodies were expected to recognize both ORFs. No specific reactivity was seen with the preimmune serum (Fig. 5A, lanes 1–3). As expected, the purified Ra8.1A-NP rabbit IgG antibodies reacted strongly with the 52–54-kDa GST-K8.1A fusion protein (Fig. 5B, lane 3) but not with the K8.1B fusion protein (Fig. 5B, lane 2) or GST alone (Fig. 5B, lane 1). In contrast, purified Ra 8.1A-CP rabbit IgG antibodies reacted strongly with both K8.1A and K8.1B fusion proteins (Fig. 5C, lanes 2 and 3) but not with the GST protein (Fig. 5C, lane 1). The reactivities of rabbit sera further verified the AA sequences of the cDNA clones encoding K8.1A and K8.1B and their expression in the GST system.

## Reactivities of human sera with ORF8.1A expressed in GST-expression system

Reactivities of human sera were next tested with the unpurified GST K8.1A and K8.1B fusion proteins (Figs. 5D–5F). No specific reactivity was seen when the blots were tested only with the alkaline phosphatase-labeled anti-human antibodies (Fig. 5D, lanes 1–3). In contrast, sera from HIV+KS+ and HIV+KS− individuals recognized the K8.1A and K8.1B fusion proteins but not the GST protein (Figs. 5E and 5F). Although these reactivities were specific, this method was not used further to test

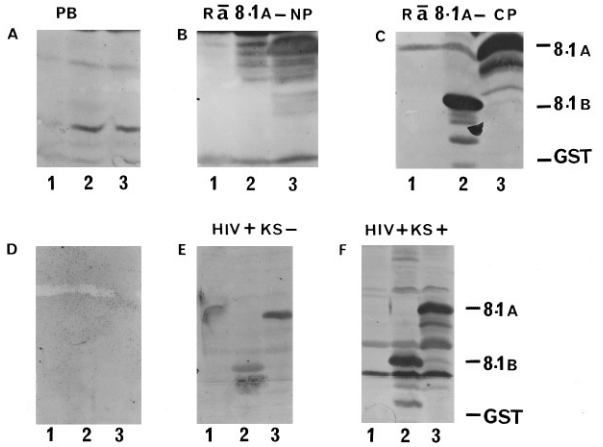


FIG. 5. Western blot reactions with total bacterial cell extracts containing GST and GST-ORFs 8.1A and 8.1B. (Lanes 1–3) Total bacterial cell extracts expressing GST only, GST-ORF K8.1B, and GST-ORF K8.1A, respectively. All rabbit sera were tested at 1:500 dilution. (A) Reactivities of the preimmune rabbit serum. (B) Reactivities of rabbit anti-K8.1A-NP antibodies raised against synthetic peptide (AA 93–111) corresponding to the amino terminus of ORF K8.1A not present in ORF K8.1B. (C) Reactivities of rabbit anti-K8.1A-CP antibodies raised against synthetic peptide (AA 140–156) corresponding to the carboxyl termini of ORFs K8.1A and K8.1B. (D) Reactivities of alkaline phosphatase-labeled anti-human antibodies. (E) Reactivities of a serum (1:640 dilution) from HIV+KS− individual. (F) Reactivities of a serum (1:640 dilution) from HIV+KS+ individual. The locations of GST, ORF 8.1A, and ORF 8.1B are indicated.

human sera due to the presence of other bacterial proteins in these whole-cell extracts.

The GST-ORF K8.1A fusion protein and the GST alone were further purified by passing through the Glutothione Sepharose 4B, and the purity was verified by Coomassie Blue stain of the gel (Fig. 6A) and by the reactivities of purified Ra 8.1A-NP rabbit antibodies (Fig. 6B). Approximately equal quantities of these proteins (100 ng/10 $\mu$l) were loaded per lane. The purified GST protein was always localized below the 29-kDa marker (Fig. 6A, lane 1). The GST-ORF K8.1A protein was ~52–54 kDa, and the additional lower-molecular-weight proteins detected probably represent proteolytic products (Fig. 6A, lane 2). A high-molecular-weight protein of ~72 kDa also was detected and could represent a dimeric aggregate of GST-ORF K8.1A protein (Fig. 6A, lane 2). The rabbit anti-peptide antibodies (K8.1A-NP) recognized the 52–54-kDa GST-ORF K8.1A protein and the high-molecular-weight protein in Western blot reactions but not the GST protein (Fig. 6B, lanes 1 and 2). To test the reactivities of sera with the GST and GST-ORF K8.1A proteins, we used a panel of sera that were previously tested for HHV-8 antibodies by IFA with TPA-induced BCBL-1 (HHV-8+) cells, with TPA-induced BJAB cells, and with TPA-induced EBV producer cells P3HR-1 (Chandran *et al.,* 1998). Specific bright cytoplasmic fluorescence with ~30% of TPA-induced BCBL-1 cells were considered as recognizing lytic HHV-8 antigens. Although sera were tested with a starting dilution of 1:10, several sera showed nonspecific fluorescence at 1:10 and 1: 20 dilutions with BCBL-1 and BJAB cell. Hence, sera showing a reactivity at a dilution of ≥1:40 were considered positive. These sera were tested at a dilution of 1:40 with the GST and GST-ORF K8.1A proteins by Western blotting, and positive sera were retested by titration.

The sera were coded and tested in the Western blot reactions in a blind manner. Several sera showed very specific reactivities with the ORF-K8.1A protein but not with the GST proteins (representative examples are shown in Figs. 6C and 6D). Sera from 62 HIV+KS+ patients were tested, and 60 (97%) of them showed specific reactivities with ORF K8.1A fusion protein (Table 1). The titer of these sera in these Western blot reactions ranged from 1:160 to 1:10,240 (an example is shown in Fig. 6C, lanes 1–6). All the sera that tested positive by Western blotting were also positive for HHV-8 antibodies by IFA with TPA-induced BCBL-1 cells (Table 1). Among the 38 sera from HIV+KS− patients tested, 23 (61%) showed reactivities with the GST-ORF K8.1A fusion protein, and the titer of these sera ranged from 1:40 to 1:2560. Initial testing of HIV+KS− sera at a dilution of 1:40 dilution of sera showed varying degrees of GST-ORF K8.1A band intensity (Fig. 6D, lanes 1–6). This probably reflected the titer of these sera. All the HIV+KS− sera

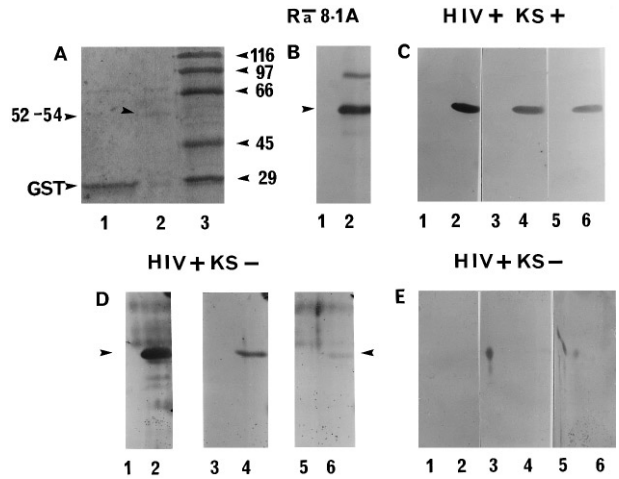


FIG. 6. Reactivities of human sera with column purified GST-ORF 8.1A. (A) GST and GST-ORF K8.1A fusion protein were purified by passing through the Glutathione Sepharose 4B, and the purity was verified by Coomassie Blue stain of the gel. Samples were analyzed on 12% acrylamide cross-linked with DATD. (Numbers) Approximate molecular mass (in kDa). (Lane 1) Column-purified GST. (Lane 2) Column-purified GST-ORF K8.1A. (Lane 3) Molecular weight markers. (Arrows) Location of GST and the GST-ORF K8.1A fusion protein. (B–E) Reactivities of sera in Western blot reactions. (Lanes 1, 3, and 5) Column-purified GST. (Lanes 2, 4, and 6) Column-purified GST-ORF K8.1A. (B) Reactivities of rabbit anti-K8.1A-CP peptide antibodies (1:500 dilution). (C) Reactivities of an HIV+KS+ serum at a dilution of 1:640 (lanes 1 and 2), 1:1280 (lanes 3 and 4), and 1:2560 (lanes 5 and 6). (D and E) Reactivity of HIV+KS− sera at a dilution of 1:40. (Arrow) Location of GST-ORF K8.1A fusion protein.

TABLE 1

Reactivities of Human Sera with HHV-8 ORF K8.1A

| Sera from | Number tested | Number + (%) | |
|---|---|---|---|
| | | ORF K8.1A WB[a] | BCBL-IFA[b] |
| HIV+KS+ patients (USA) | 62 | 60/62 (97%) | 60/62 (97%) |
| HIV+KS− gay men (USA) | 38 | 23/38 (61%) | 23/38 (61%) |
| Healthy adult men (HIV−) (USA) | 120 | 9/120 (8%) | 9/120 (8%) |
| Children (1–5 yr) (USA) | 48 | 0/48 | 0/48 |

[a] Sera were tested at a starting dilution of 1:40 with Western blotted GST and GST-ORF K8.1A protein.

[b] Sera were tested at a starting dilution of 1:10 with TPA-induced HHV-8+ BCBL-1 cells and TPA-induced HHV-8− BJAB cells (Chandran *et al.,* 1998).

that were positive by Western blotting were also positive for HHV-8 lytic antibodies by IFA (Table 1).

Sera from HIV+KS− patients that were negative for HHV-8 antibodies by IFA were also negative when tested with the ORF K8.1A protein (Fig. 6E, lanes 1–6). Similarly, sera from 48 children (ages 1–5) were negative both in IFA and in Western blots (Table 1). We also tested 120 sera from age-matched (18–50 years) HIV− healthy adult men, and among these, 45 were from blood bank donors and 75 were from adult men from the general population. The sexual orientation of these individuals is not known. Only 9 (8%) of the 120 HIV− men were positive for HHV-8 lytic antibodies by IFA. All 9 sera were also positive in Western blot reactions, and their antibody titers ranged from 1:40 to 1:160. (Table 1). Three (3 of 45, or 6.6%) of these positive sera were from blood bank donors, and six (6 of 75, or 8%) were from the other adult male groups. These results suggested that testing the antibodies against ORF K8.1A protein provide a specific HHV-8 serological assay.

## DISCUSSION

Our earlier studies showed that human humoral responses were directed against a number of HHV-8 proteins (Chandran *et al.,* 1998). Sera from HIV−KS− individuals and from a majority of HIV+KS− individuals had low titers of HHV-8 antibodies, recognizing only a subset of viral proteins (Chandran *et al.,* 1998). Hence, it is critical to use immunogenic antigens detecting the low level of antibodies, presumably the persisting antibody responses, to study the HHV-8 seroprevalence. HHV-8 ORF 73 is identified as being responsible for the majority of the LANA-type reactivities of sera (Rainbow *et al.,* 1997). Simpson *et al.,* (1996) and Lin *et al.,* (1996) identified the HHV-8 ORF65 protein as an immunogenic lytic cycle associated protein. However, not all HIV+KS+ sera showed positive reactions with the ORF 65 protein, suggesting that a more immunodominant antigen needs to be used. The initial identification of cDNA encoding the HHV-8 ORF K8.1A and K8.1B by screening the cDNA library with an HIV+KS+ serum suggested that these proteins encoded immunogenic domains. We selected the ORF K8.1A for further testing with sera because it possesses AA not present in ORF K8.1B. Our results showed a high degree of concordance between IFA detecting HHV-8 lytic antibody responses and Western blot reactions detecting anti-ORF K8.1A antibodies. All sera that were seropositive by IFA with TPA-induced BCBL-1 cells were also positive for anti-ORF K8.1A antibodies. Although the IFA using TPA-induced BCBL-1 cells is specific for HHV-8 and detects a spectrum of responses against the various HHV-8 antigens, it is tedious, subjective, and hampered by nonspecific reactions at lower dilutions of sera. The ORF K8.1A is specific for HHV-8, suggesting that this protein can be used to develop a sensitive and specific assay to measure HHV-8 seroprevalence in the population. We have cloned and expressed the ORF K8.1A and K8.1B, ORF 73, and ORF 65 in baculovirus system, and attempts are on the way to develop a rapid comparative ELISA measuring lytic and latent antibodies.

Our results showed that the HHV-8 ORF K8.1 gene encodes glycoproteins generated from spliced transcripts. The Northern blot hybridization kinetic experiments suggested that these transcripts belong to the truly late class transcript. Analysis of HHV-8 genomic sequences show that like other herpes viruses, HHV-8 encodes for several glycoproteins. We have shown that HHV-8-specific glycoproteins are good targets for human humoral immune responses (Chandran *et al.,* 1998). From BCBL-1 cells, HHV-8 antibody-positive human sera recognized several glycoproteins with an approximate molecular weights of 128,000, 116,000, 105,000, 80,000, 75,000, 65,000, 60,000–55,000, 50,000, 45,000, 41,000–38,000, and 34,000–32,000. These glycoproteins are probably encoded by the HHV-8 ORF K1, 8, 22, 47, and K8.1 genes (Neipel *et al.,* 1997; Russo *et al.,* 1996). The identity of the individual glycoproteins encoded by these genes, including the ORF K8.1A and K8.1B, must be determined. Preliminary studies with anti-ORF K8.1A and K8.1B antibodies show that ORF 8.1A and 8.1B glycoproteins are present in the HHV-8 virion envelopes, and the broader glycoproteins in the 45,000–38,000 regions recognized by human sera (Chandran *et al.,* 1998) are encoded by the HHV-8 ORF K8.1A and K8.1B (data not shown). Further characterization of these glycoproteins will be presented elsewhere.

Among the different glycoproteins encoded by HHV-8, ORFs 8, 22, and 47 are homologous to the glycoproteins gB, gH, and gL, respectively, which are conserved among the other herpes viruses. In contrast, comparisons with the human or animal herpes viruses sequences to date show that the ORFs K8.1A and K8.1B are unique for HHV-8. This suggests that ORF K8.1A and

K8.1B glycoproteins may be mediating important biological function or functions specific for HHV-8. The location of the HHV-8 ORF K8.1A gene in the genome strengthens such a notion. The HHV-8 K8.1A gene is positionally colinear to the EBV gene encoding the major EBV glycoproteins gp350/gp220 (Kieff *et al.,* 1996; Neipel *et al.,* 1997). Similar to HHV-8 ORF K8.1B, the EBV gp220 arises from an in-frame deletion of gp350 gene (Kieff *et al.,* 1996). The gp350 and gp220 are highly glycosylated with several N- and O-linked sugar attachments (Kieff *et al.,* 1996). In addition to the potential *N*-glycosylation sites, the HHV-8 ORFs K8.1A and K8.1B possess a high number of serine and threonine residues (28 and 20, respectively), which are the potential sites for *O*-glycosylation. Studies are in progress to determine the nature of sugars in the ORF K8.1A and K8.1B. The EBV gp350 and gp220 are responsible for the specific B-cell tropism of the virus in that they are the first viral contact protein mediating the binding to the receptor for complement fragment C3d (Kieff *et al.,* 1996). HHV-8 also infects B-cells (Boshoff *et al.,* 1995; Rennne *et al.,* 1996), and it is not clear whether it uses the same receptor as EBV. Because HHV-8 DNA and transcripts were shown to be present in macrophages (Orenstein *et al.,* 1997) and endothelial cells (Staskus *et al.,* 1997), HHV-8 may have broader tropism than EBV. Further studies are in progress to define the HHV-8 ORF 8.1A and 8.1B glycoproteins in the virions; the role of these two different proteins in HHV-8 biology, such as binding and entry into cells; and the ability of anti-K8.1A and -K8.1B antibodies to neutralize the infectivity.

## MATERIALS AND METHODS

### Cell lines

HHV-8+ and EBV− body cavity B-cell lymphoma cell line BCBL-1 (a gift from Dr. McGrath, University of California at Los Angeles) (Renne *et al.,* 1996) was grown in RPMI 1640 medium with glutaMAX I (GIBCO BRL, Grand Island, New York) supplemented with 10% heat-inactivated fetal bovine serum and antibiotics.

### Serum samples and IFA

Sera from HIV+KS+ and HIV+KS− homosexual adult male patients (ages 18–50), age-matched HIV−KS− adult men, and children (ages 1–5) were used in these studies (Chandran *et al.,* 1998; Smith *et al.,* 1997). All sera were stored at −20°C and heat inactivated at 56°C for 30 min before use. IFA with TPA-induced BCBL-1 cells was performed as described previously (Chandran *et al.,* 1998; Smith *et al.,* 1997).

### Construction of cDNA library and screening of ZAPII expression system

BCBL-1 cells were induced with 20 ng/ml TPA (Sigma Chemical Co., St. Louis, Missouri), and aliquots were collected at 24, 48, 72, and 96 h postinduction. For reverse transcription (RT)–PCR, total RNA was extracted from these cells, treated with RQ1-DNase (Promega, Madison, Wisconsin), and verified for the absence of HHV-8 DNA by DNA-PCR using ORF 25 and 26 primers (Smith *et al.,* 1997). The mRNA was isolated by Oligotex mRNA isolation kit (Qiagen, Chatsworth, California) and used in cDNA synthesis using oligo(dT) linker-primer containing *Xho*I site (Stratagene, La Jolla, California) according to the manufacturer's recommendations. Synthesized cDNAs were tested for HHV-8 messages by DNA-PCR using HHV-8 ORFs 22, 25, and 26 primers as described previously (Smith *et al.,* 1997), and appropriate-size fragments were detected. The cDNA library in the ZAPII expression system was made according to the manufacturer's recommendations. After amplification, this library had a titer of $\sim 4 \times 10^9$ pfu/ml with 99% recombinant phages.

More than 100,000 plaques from this library were screened with an HIV+KS+ serum (IFA titer, 1:10,240) using procedures described previously (Chang and Balachandran, 1991). To remove nonspecific reactivities, the serum was absorbed with HHV-8− control B-cells (BJAB) and with bacterial lysates containing nonrecombinant phages. The absorbed serum was diluted 1:6000 in the blocking buffer (Chang and Balachandran, 1991) and used to screen the library. Immunoreactive phages were picked and purified by four subsequent steps of screening. After four screenings, 56 clones were isolated. The identified cDNAs were released into the phagemid forms by *in vivo* excision using the ExAssist helper phage.

### 5′ RACE

The sequences of the isolated K8.1A and K8.1B cDNA clones and the splicing events were verified by 5′ RACE. This was done by using the Marathon cDNA amplification kit (Clontech, Palo Alto, California) and the RNA extracted from TPA-induced BCBL-1 cells (48 h postinduction). Single-stranded cDNAs were synthesized using Moloney murine leukemia virus–RT and an oligo(dT) primer provided with the kit. The second strand was synthesized, and double-stranded adapters were ligated to both ends of the cDNA molecules. These cDNAs were amplified by PCR using an adapter-specific primer provided in the kit and with an HHV-8-specific primer corresponding to the ORF K8.1A and K8.1B 3′-end stop site (5′-ATGGTTTTGTGTTACACTATGTAGGG-3′). These double-stranded cDNA PCR products were ligated into the PGEM-T vector.

### DNA sequence analysis

The cDNA clones (both orientations) were sequenced in the Biotechnology Center at the University of Kansas Medical Center. Sequences were analyzed and compared with the data bank by BLAST program. The two

ORFs encoded by the cDNAs were designated as HHV-8 ORF K8.1A and K8.1B.

### Raising rabbit antibodies against synthetic peptides recognizing HHV-8 ORF K8.1A and K8.1B

To raise anti-peptide antibodies, two peptide regions in the HHV-8 ORF K8.1A AA sequences were selected based on the hydropathic (Kyte–Doolittle method), antigenic (Jameson–Wolf method), and surface probability (Emini method) profiles. The peptide designated K8.1A-NP (TSASGSGEDAIDESGSGEE- AA 93–111) corresponds to the amino terminus of the HHV-8 ORF K8.1A. This stretch of AA is not present in the ORF K8.1B due to a splicing event resulting in an in-frame deletion. The peptide designated K8.1A-CP (FSGSYSSGEPSRTTRIR-AA 140–156) corresponds to the carboxyl terminus of HHV-8 ORFs K8.1A and K8.1B and is absent in the genomic HHV-8 ORF K8.1. These two peptides were synthesized over a core of poly-L-lysine in the Biotechnology Center. New Zealand White male rabbits were first immunized subcutaneously and intramuscularly with 100 $\mu$g of peptides in Freund's complete adjuvant. Twenty and 40 days later, rabbits were immunized with the same amount of antigens in Freund's incomplete adjuvant. The rabbits were boosted 2 months after the last injection and were bled 10 days later. IgG antibodies (Ra 8.1A-NP and Ra8.1A-CP) were purified from the sera by affinity chromatography on a Protein A–agarose column.

### Construction and expression of HHV-8 ORF K8.1A and K8.1B in the GST gene fusion system

HHV-8 ORFs K8.1A and K8.1B from the cDNAs were amplified using 5′ primer (cggggatccatgagttccacacagattcgc with a *Bam*HI site) and 3′ primer (atggtctcgagttacactatgtagggtttc with an *Xho*I site). Amplified PCR fragments were purified and ligated into pGEM-T vector (Promega). Orientation was verified by restriction enzyme digestion and sequence analysis. These plasmids were digested with *Bam*HI and *Xho*I and inserted into the GST expression vector pGEX-4T-1 (Pharmacia, Piscataway, New Jersey). Orientation was verified by restriction enzyme digestion. Induction of fusion protein was done by adding IPTG (1 mM) to bacterial cultures grown to an $OD_{600}$ of 0.2–0.5. The induced GST and GST-ORF K8.1A fusion proteins were purified by Glutathione Sepharose 4B (Pharmacia) according to the manufacturer's recommendations.

### *In vitro* transcription and *in vitro* translation of cDNA

*In vitro* transcription of the HHV-8 ORF K8.1A and K8.1B cDNA inserts in pGEM-T was done by linearizing the plasmid with appropriate restriction enzymes and synthesizing sense RNA transcripts using Sp6 polymerase. Capping of RNA at 5′ was carried out according to procedures described in the Riboprobe transcription system instruction manual (Promega). RNA samples from *in vitro* transcription experiments were translated *in vitro* using [$^{35}$S]methionine and rabbit reticulocyte lysate preparations (Promega) with and without microsomal membranes according to the manufacturer's recommendations. *In vitro* translated products were boiled in sample buffer, and equal trichloroacetic precipitable counts were analyzed by SDS–PAGE. The *in vitro* synthesized products in the presence of membranes were treated with *N*-glyconase according to the procedures of Balachandran and Hutt-Fletcher (1995).

### Western blot reactions

Approximately 100 ng of each protein in a 10-$\mu$l volume was loaded per lane in the SDS–PAGE minigel apparatus (BioRad, Hercules, California). Protein samples were separated in 12% acrylamide cross-linked with DATD and electrophoretically transferred to nitrocellulose sheets (Chang and Balachandran, 1991). Standard molecular weight markers (Sigma) were included in parallel lanes. After the transfer, the membranes were stained with Ponceau-S for 5 min, and the positions of the transferred proteins and molecular weight markers were marked. The stain was washed by distilled water. The nitrocellulose membranes were subsequently incubated overnight with blocking buffer (10 mM Tris–HCl, pH 7.2, 0.15 M NaCl, and 5% skim milk) and then reacted with 2–4 ml of blocking buffer containing appropriate dilutions of rabbit antisera or human sera for 3 h at room temperature. The membranes were washed with washing buffer (10 mM Tris–HCl, pH 7.2, 0.15 M NaCl, and 0.3% Tween 20), and finally incubated for 1 h with alkaline phosphatase-conjugated goat anti-rabbit IgG or anti-human IgG antibodies (HyClone Laboratories). After washing, the bound antibodies were detected using 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium substrate (Sigma).

### RNA extraction, riboprobe synthesis, and Northern blot hybridization

Total RNA was isolated from uninduced and TPA-induced BCBL-1 cells and BJAB cells with and without PAA treatment as described previously (Chan *et al.,* 1998). The RNA samples were analyzed by Northern blot hybridization with antisense RNA probes transcribed from the HHV-8 ORF K8.1A and 59 cDNA (Chan *et al.,* 1998) using the Riboprobe kit (Promega). Northern blots were performed on nylon membranes using standard methodology with 10 $\mu$g of total RNA for each lane. Blotted membranes were hybridized overnight at 65°C in 5 ml of RNA hybridization buffer (5× Denhardt's reagent, 5× SSPE, 0.5% SDS, 50% formamide, and 100 $\mu$g/ml salmon sperm DNA) containing $\sim 1 \times 10^7$ cpm of radiolabeled *in vitro* transcribed RNA. After hybridization, membranes were washed as per standard procedures and exposed to Kodak XAR5 film at

−70°C. The membranes were stripped and then rehybridized with a GAPDH riboprobe and a riboprobe from a cDNA (HHV-8 ORF 59) encoding HHV-8 early-late protein P50 (Chan *et al.,* 1998).

## Nucleotide sequence accession numbers

The nucleotide sequences reported here have been deposited in the GenBank database and assigned accession numbers AF068829 and AF068830.

## ACKNOWLEDGMENTS

This study was supported in part by U.S. Public Health Service Grants AI-33502, AI-30356, CA-75911, and P20-CA57129 and by Grant KUMCRI-8906. Technical assistance from Dongmei Wang is greatly acknowledged.

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