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Open Reading Frame 26 of Human Herpesvirus 8 Encodes a Tetradecanoyl Phorbol Acetate- and Butyrate-Inducible 32-Kilodalton Protein Expressed in a Body Cavity-Based Lymphoma Cell Line

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DNA sequences corresponding to a novel herpesvirus (human herpesvirus 8 [HHV8]) are associated with Kaposi's sarcoma (KS), Castleman's disease, and body cavity-based lymphomas (BCBL). Studies of a BCBL-derived cell line suggest a direct correlation between seropositivity against antigens specifically present in such lines and the development of KS. We have generated recombinant proteins corresponding to open reading frame (ORF) 26 of HHV8 and have produced affinity-purified antibodies. Using these antibodies, we studied the expression of HHV8 ORF26 in a BCBL-derived cell line and found that it encodes a cytoplasmic protein whose expression is induced 16-fold by treatment with phorbol ester or sodium butyrate. This protein induction correlates with a significant induction of viral RNA transcripts. Interestingly, under our experimental conditions minimal increases in viral DNA were observed. No antibodies to the ORF26 protein of HHV8 were found in the sera from two human immunodeficiency virus-positive patients with KS as determined by immunoprecipitation analysis. However, antibodies in the sera from the two KS patients immunoprecipitated a 34-kDa protein found in extracts from induced BCBL1 cells that was not recognized by the control sera.

Kaposi's sarcoma (KS) was first recognized in middle-aged European men of Mediterranean origin (classical KS), but it has also been reported in African men (African or endemic KS), in patients with transplant-associated immunosuppression, and in persons infected with human immunodeficiency virus (HIV; epidemic KS) (18). This neoplasm is 20,000 times more common among persons with AIDS than in the general population and is 300 times more common among AIDS patients than in other immunocompromised groups (3). Among HIV-infected people, homosexual men are 10 times more likely to develop KS than are other groups (2). The lifetime risk of KS in homosexuals with AIDS is 50% (2). In all affected groups, the malignancy is characterized by multifocal, polyclonal tumors. These lesions are heterogeneous in composition, but spindle-shaped endothelial cells mixed with cells of dendritic and monocytic origin predominate (26). When associated with AIDS, KS is particularly aggressive, resulting in angiogenesis, inflammatory cell infiltration, and edema (17). KS may be a cytokine-mediated disease, and a role for HIV Tat in the induction of the aggressive form of KS in AIDS patients has been postulated (10).

Adoption of "safe sex" practices correlates with a reduced incidence of KS in HIV-infected individuals and suggests the involvement of an infectious agent (18). In addition, DNA sequences corresponding to human herpesvirus 8 (HHV8) have been found in KS lesions (1, 4, 7–9, 14, 20, 22, 24, 25, 32). These findings, together with recent epidemiological data (13, 16), support the theory of an infectious etiology (3, 18, 29). The viral sequences identified showed strong homology with minor capsid proteins of other gammaherpesvirus subfamily members including BDLF1 of Epstein-Barr virus (EBV) and the open reading frame 26 (ORF26) protein of herpesvirus saimiri (7, 21, 30).

HHV8 viral sequences have also been found associated with body cavity-based lymphomas (BCBL) occurring in HIV-positive patients (5, 6, 11, 15, 23). BCBL invade the pleural or peritoneal cavities where they grow as ascites tumors (5, 27). BCBL-derived cell lines provide an in vitro model system in which to study this novel herpesvirus and have begun to provide data regarding the seroprevalence of antibodies against HHV8 (12, 13, 16, 19). In these studies, sera from KS patients were screened (by Western blot or immunofluorescence analysis) for antibodies specific to putative HHV8 antigens expressed in BCBL-derived cell lines. The results suggested a direct correlation between seropositivity against these putative HHV8 antigens and the presence (or eventual development) of KS. However, although the antigens were specific for BCBL-derived cell lines, their identities are unknown. In an effort to characterize specific HHV8 proteins and to evaluate the presence of antibodies specific for known HHV8 proteins in human sera, we have generated recombinant fusion proteins corresponding to ORF26 of HHV8 and affinity-purified antibodies that recognize the HHV8 ORF26 gene product. We used these reagents and the recently described BCBL1 cell line (16, 27, 28) to study the expression of HHV8 ORF26. Our results show that BCBL1 cells express low basal levels of the ORF26-encoded protein which are induced after tetradecanoyl phorbol acetate (TPA) or sodium butyrate treatment. Southern blot and fluorescent in situ hybridization (FISH) analysis showed a minimal increase in the levels of viral DNA. Instead, the increased protein levels correlated with a significant increase of HHV8-specific transcripts. Interestingly, sera from two HIV-infected individuals with KS and pooled sera from healthy individuals showed no antibodies specific for the HHV8 ORF26 protein, as determined by immunoprecipitation analysis. However, antibodies in the sera from the two KS patients immunoprecipitated a 34-kDa protein found in extracts from

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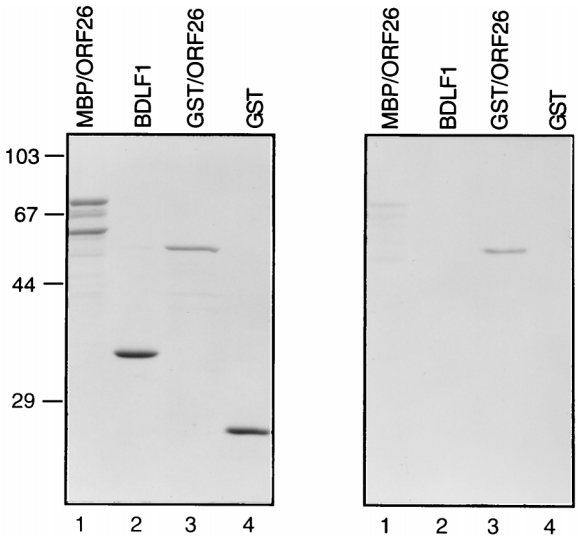


FIG. 1. Characterization of recombinant proteins and antibodies. Proteins separated by SDS-PAGE were either stained for visualization (left panel) or transferred onto nitrocellulose membranes and immunoblotted with affinity-purified anti-HHV8 ORF26 protein antibodies (right panel). Lanes (both panels): 1, MBP-ORF26 fusion protein; 2, histidine-tagged BDLF1; 3, GST-ORF26 fusion protein; 4, GST. For the left panel, 4 μg of each protein was loaded. For the Western blot analysis (right panel) the amounts of protein were decreased to 5 ng for lane 1 and to 150 ng for lanes 2 to 4.

induced BCBL1 cells which was not recognized by the control sera.

MATERIALS AND METHODS

Expression of recombinant proteins. The coding sequence for ORF26 of HHV8 was obtained through PCR amplification of KS-derived DNA by using previously published primers (25). The PCR product was cloned into the pCRII vector (Invitrogen, San Diego, Calif.), resulting in the construct pCORF26. A fusion protein combining the maltose-binding protein (MBP) and the ORF26 protein of HHV8 (MBP-ORF26 fusion protein) was generated by inserting a 933-bp *Hpa*I-*Eco*RI fragment from pCORF26 downstream of the MBP ORF of the pMAL vector (New England Biolabs, Beverly, Mass.). The resulting construct was expressed in bacteria and purified over an amylose resin column. In addition, a glutathione *S*-transferase (GST)-ORF26 fusion protein was generated by ligating the *Hpa*I-*Eco*RI ORF26 fragment downstream of the GST ORF in the pGEX4T2 vector (Pharmacia Biotech, Piscataway, N.J.). This construct was expressed in bacteria, and the protein product was purified over a glutathione affinity column. The BDLF1 ORF was obtained by PCR amplification of a portion of the EBV *Bam*D subgenomic fragment (kindly provided by Clare E. Sample), followed by cloning into pCRII (Invitrogen), resulting in pBDLF1. An *Xho*I-*Eco*RI fragment of pBDLF1 was then subcloned into the vector pET28a (Novagen, Madison, Wis.). The resulting construct was expressed in bacteria, and the polyhistidine-tagged BDLF1 protein was purified over a nickel affinity column. The sequences of both the BDLF1 ORF and HHV8 ORF26 were confirmed by DNA sequencing.

Generation of antibodies against the ORF26 protein of HHV8. Purified MBP-ORF26 fusion protein was used to immunize naive New Zealand White rabbits (Rockland, Gilbertsville, Pa.). Rabbit antisera were run over an MBP affinity column to remove most of the MBP-specific antibodies, and the flowthrough was passed over an MBP-ORF26 affinity column. The eluate from the affinity column was analyzed for the presence of antibodies to the ORF26 protein of HHV8 by Western blot and immunoprecipitation analyses (described below).

Cell lines and culture conditions. Human embryonic kidney 293T cells which express the large T antigen of simian virus 40 (kindly provided by G. R. Kitchingman) were cultured in Dulbecco's modified Eagle's medium (Fisher, Pittsburgh, Pa.) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, Utah), 50 IU of penicillin per ml, and 50 μg of streptomycin per ml. These cells were maintained at 37°C in a humidified atmosphere with 10% CO_2. The BCBL1 cell line was derived from a BCBL (kindly provided by D. Ganem and M. McGrath through the AIDS repository) from an HIV-positive patient (27). This cell line was cultured in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 5×10^{-5} M 2-mercaptoethanol, 50 IU of penicillin per ml, and 50 μg of streptomycin per ml. These cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2.

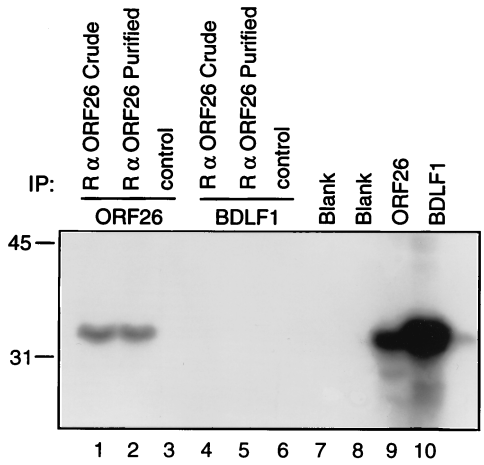


FIG. 2. In vitro transcription and translation of HHV8 ORF26 and immunoprecipitation of HHV8 ORF26 protein. Translated products (HHV8 ORF26 protein, lanes 1 to 3 and 9; EBV BDLF1, lanes 4 to 6 and 10) were immunoprecipitated with anti-HHV8 ORF26 protein serum (lanes 1 and 4), affinity-purified anti-HHV8 ORF26 protein antibodies (lanes 2 and 5), or control antibodies (lanes 3 and 6). HHV8 ORF26 protein and BDLF1 translation products used for the immunoprecipitations were loaded in lanes 9 and 10, respectively.

In vitro transcription and translation. Full-length mRNA corresponding to HHV8 ORF26 was transcribed from pCORF26 by using the mMessage mMachine kit (Ambion, Austin, Tex.). We followed the manufacturer's instructions to translate the transcripts in rabbit reticulocyte lysates (Promega, Madison, Wis.) with 30 μCi of Tran-[^{35}S]-label (ICN, Costa Mesa, Calif.) (total reaction volume of 50 μl). The reaction was terminated by adding RNase A (10 μg) and incubating for 5 min at room temperature.

For immunoprecipitation, the in vitro-translated products were solubilized in lysis buffer (0.5% Nonidet P-40, 20 mM Tris [pH 8], 0.15 M NaCl, 2 mM EDTA, 1 μg of aprotinin per ml, 10 μg of leupeptin per ml, 1 mM phenylmethylsulfonyl fluoride) and incubated with rabbit anti-HHV8 ORF26 protein antibodies (or the appropriate control antisera) for 1 h at 4°C. The immunoadsorbed products were then incubated with 70 μl of 50% (wt/vol) Sepharose 4B-protein A beads for 1 h at 4°C. The immunoprecipitates were washed once with lysis buffer, twice with 0.5 M $MgCl_2$, and then twice with lysis buffer. To release the immune complexes, the beads were incubated in sodium dodecyl sulfate (SDS) sample buffer (10% glycerol, 10% SDS, 10% 2-mercaptoethanol, 0.1 M Tris [pH 6.8], 0.1% bromophenol blue) for 10 min at 95°C. The samples were resolved by SDS–12% polyacrylamide gel electrophoresis (PAGE) and were visualized by autoradiography.

Metabolic labeling. BCBL1 cells were induced with TPA (20 ng/ml) for 36 h. Induced and uninduced cells were starved for 30 min in methionine- and cysteine-free RPMI 1640 (ICN) supplemented with L-glutamine, sodium pyruvate, and antibiotics as described above. Cells were metabolically labeled for 3 h at 37°C in the same medium containing 10% dialyzed FBS and 500 μCi of Tran-[^{35}S]-label (ICN) per 3×10^7 cells. After being labeled, cells were harvested, washed in ice-cold Dulbecco's phosphate-buffered solution (D-PBS; Fisher), and lysed in 2.5 ml of lysis buffer. Metabolically labeled products were immunoprecipitated as described above by incubation for 1 h at 4°C with rabbit anti-HHV8 ORF26 protein antibodies, heat-inactivated human sera from HIV-infected KS patients, rabbit anti-rat immunoglobulin G (IgG), or human sera from healthy persons. Sepharose 4B-protein A beads were subsequently added, and samples were incubated for 1 h at 4°C. The immunoprecipitates were then washed and examined by SDS-PAGE as described above.

Western immunoblot analysis. Proteins separated by SDS-PAGE as described above were transferred to Hybond-C nitrocellulose membranes (Amersham Life Science, Arlington Heights, Ill.) by using a semidry electrotransfer apparatus (Bio-Rad, Hercules, Calif.). After being blocked with 5% nonfat dry milk in TBST (Tris-buffered saline plus Tween 20: 10 mM Tris [pH 8], 0.15 M NaCl, 0.05% Tween 20) for a minimum of 1.5 h, membranes were probed with affinity-purified rabbit anti-HHV8 ORF26 protein antibodies (1:100 in 5% dry milk–TBST) for 1 h at room temperature. The blots were washed three times (5 min for each wash) with TBST at room temperature. Bound anti-HHV8 ORF26 protein antibodies were detected by incubation for 1 h at room temperature with alkaline phosphatase-conjugated anti-rabbit IgG (1:4,000; Promega) or horseradish peroxidase-conjugated anti-rabbit IgG (1:50,000; Amersham). To visualize the bound complexes, 5-bromo-4-chloro-3-indolyl-1-phosphate and nitroblue tetrazolium were added to the alkaline phosphatase conjugates; horseradish peroxidase conjugates were visualized by using an enhanced chemiluminescence system (Amersham).

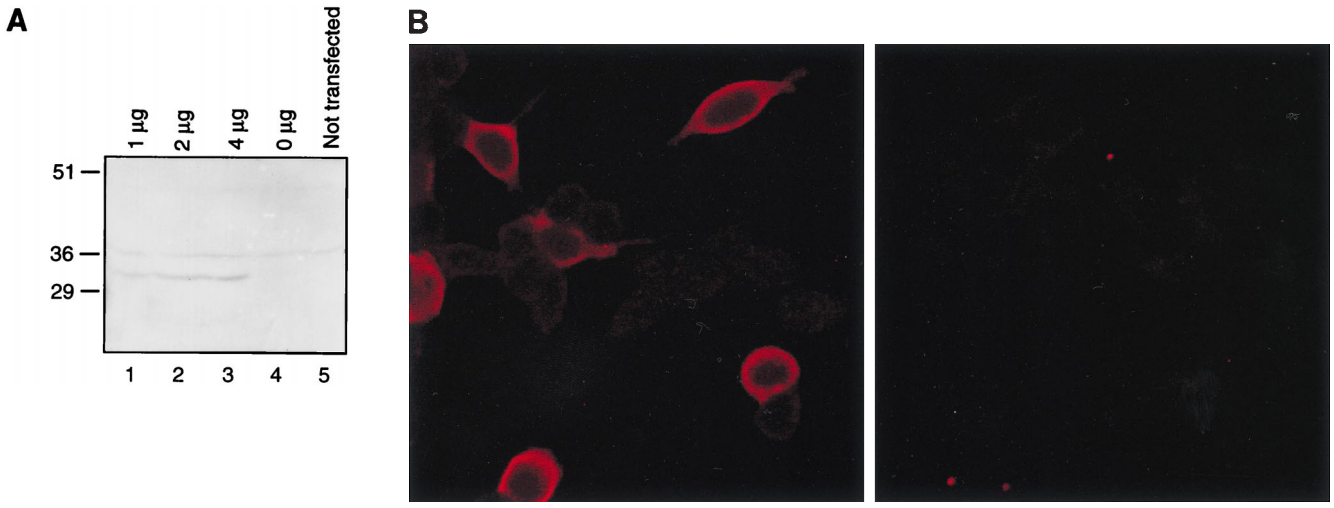


FIG. 3. Expression of HHV8 ORF26 in 293T cells. (A) Western blot analysis of cells transfected with 1, 2, 4, or 0 μg of pCORF26 DNA (lanes 1 to 4, respectively). Lane 5 was loaded with extract from control 293T cells. Cell lysates corresponding to 800 μg of total protein were loaded for each sample. A specific band of 32 kDa only seen in lanes 1 to 3 corresponds to the ORF26 protein product. Alkaline phosphatase-conjugated secondary antibody was used for detection as described in Materials and Methods. (B) Immunofluorescence analysis using affinity-purified anti-HHV8 ORF26 protein antibodies (left panel) or control affinity-purified rabbit anti-rat IgG (right panel). FITC-labeled goat anti-rabbit IgG was used for immunodetection. Only cytoplasmic staining was observed. Magnification, ×416.

Transient transfections. For these experiments, ORF26 of HHV8 was subcloned into pCDNA, a mammalian expression vector (Invitrogen), resulting in the construct pCORF26. The day before transfection, 10^6 293T cells were plated per well in six-well plates (Falcon; Becton Dickinson, Franklin Lakes, N.J.). The transfections were performed by Lipofectamine (Life Technologies, Gaithersburg, Md.) or calcium phosphate coprecipitation with CalPhos Maximizer (Clontech, Palo Alto, Calif.). The cells were harvested at 48 h posttransfection, counted, and divided into two samples, one for immunofluorescence analysis and another for Western blot analysis. The protocol for immunofluorescence analysis is described below. For Western blot analysis samples were resuspended in 50 μl of 0.25 M Tris (pH 8) per 5×10^6 cells and frozen and thawed three times in a dry ice-ethanol bath. After the supernatants were clarified by centrifugation (15,000 × *g* for 5 min at 4°C), the total protein concentration was quantified by using the Bio-Rad protein assay. Samples were then used for Western blot analysis as described above.

Immunofluorescence analysis. Transfected 293T cells were harvested at 48 h posttransfection, plated at a density of 10^4 cells/well on 10-well (6-mm) Teflon-coated slides (Cel-Line Associates, Newfield, N.J.), and incubated at 37°C in a humidified atmosphere with 5% CO_2 for 17 h. BCBL1 cells were plated at a density of 50,000 cells per well on polylysine (Sigma, St. Louis, Mo.)-coated slides for 20 min at room temperature. The slides were rinsed in D-PBS (Fisher), and the cells were fixed in 3.7% formaldehyde for 20 min at room temperature. The slides were stored at 4°C in D-PBS containing 0.02% sodium azide until use. Prior to staining, cells were permeabilized by treatment with Triton X-100 (0.3% in D-PBS) for 15 min at room temperature. Nonspecific binding of the secondary antibodies was blocked by treating the slides with 5% normal goat serum (NGS; Sigma) at 37°C for 30 min. Cells were then incubated at 37°C for 45 min with undiluted affinity-purified anti-HHV8 ORF26 protein antibodies in 1% NGS. Affinity-purified rabbit anti-rat IgG (Jackson Immunoresearch, West Grove, Pa.) was used in parallel stainings as a negative control. After three washes with D-PBS, the slides were incubated at 37°C for 45 min with fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG (Jackson Immunoresearch; 1:800 in D-PBS containing 1% NGS). To highlight the cell nucleus, in some experiments propidium iodide was added to the primary antibody (0.2-μg/ml final concentration). Fluorescent images were obtained with a Leica DM IRBE laser scanning confocal microscope.

Northern blot analysis. Total and poly(A)$^+$-selected RNAs were isolated by acid-phenol–guanidinium thiocyanate extraction with RNA STAT-60 (Tel-Test, Friendswood, Tex.) or with the Fast Track kit (Invitrogen) according to the manufacturer's instructions. For each sample, 15 μg of total [or 3 μg of poly(A)$^+$] RNA was fractionated on a 1.5% agarose–2.2 M formaldehyde gel and blotted onto Nytran membranes by using a Turboblotter (Schleicher and Schuell, Keene, N.H.). The nucleic acids were cross-linked to the filters with a UV transilluminator (Chromato-vue model 0-63; Ultra-Violet Products, San Gabriel, Calif.). The blots were blocked (prehybridized) at 68°C for 20 min in QuikHyb solution (Stratagene, La Jolla, Calif.) prior to the addition of the radiolabeled probe. The probes used were a 971-bp *Eco*RI DNA fragment containing ORF26 of HHV8 and a 500-bp *Xba*I-*Hin*dIII DNA fragment of the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA clone (pSP64 GAPDH). The GAPDH probe served as an internal control for the amount of RNA loaded per well. In addition, a strand-specific RNA probe corresponding to the entire ORF26 of HHV8 was used to determine the orientation of the transcripts. Hybridization of the probes to the filters was done at 68°C for 1 h in QuikHyb solution containing 100 μg of denatured salmon sperm DNA per ml. Blots were briefly rinsed at room temperature in a 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) solution containing 0.1% SDS and then washed in this solution at 68°C; the wash solution was changed several times during a period of 1 to 2 h. Blots were exposed overnight to Kodak XAR-5 film with intensifying screens at −80°C. The radioactivity associated with each band was determined with a PhosphorImager (model 425F; Molecular Dynamics, Sunnyvale, Calif.).

Southern blot analysis. Genomic DNA was isolated by using a QIAamp tissue kit (Qiagen, Chatsworth, Calif.) according to the manufacturer's instructions. For each lane, 5 μg of DNA was digested with *Hin*dIII, separated on a 0.7% agarose gel, and blotted onto Nytran membranes by alkaline transfer with a Turboblotter (Schleicher and Schuell). The filters were baked at 80°C for 20 min. Hybridization and washing conditions were as described above for Northern blot analysis.

FISH analysis. FISH was performed as described previously (31). Two *Not*I fragments (11 and 4 kb) were cloned from a genomic library made from a KS lesion of an HIV-infected patient. The 11-kb insert contained the entire ORF26 of HHV8 and 10 kb of downstream viral sequences. The smaller fragment contained an additional 4 kb of contiguous downstream viral sequences. Both clones were labeled with digoxigenin dUTP by nick translation to generate HHV8-specific probes. The hybridized probes were visualized by staining with fluorescein-conjugated antidigoxigenin antibodies. As a control, we included a biotin-labeled probe specific for the EBV *Sal*IB fragment (kindly provided by John W. Sixbey); this probe was detected by staining with Texas red-conjugated avidin. BCBL1 cells were found to be negative for the presence of EBV, as previously reported (27, 28).

RESULTS

Generation of antibodies to the ORF26 protein of HHV8. To study the expression of HHV8 ORF26, several recombinant proteins were generated (Fig. 1A; see Materials and Methods). The MBP-ORF26 fusion protein was used to immunize naive New Zealand White rabbits. The rabbit antisera recognized the ORF26 protein since not only the MBP- but also a GST-ORF26 fusion protein was detected by Western blot analysis (Fig. 1B, lanes 1 and 3, respectively). The antibodies were specific for the HHV8 ORF26 protein, as they failed to recognize GST alone (Fig. 1B, lane 4) and BDLF1 (Fig. 1B, lane 2), an EBV protein 39% homologous to the ORF26 protein of HHV8 (7).

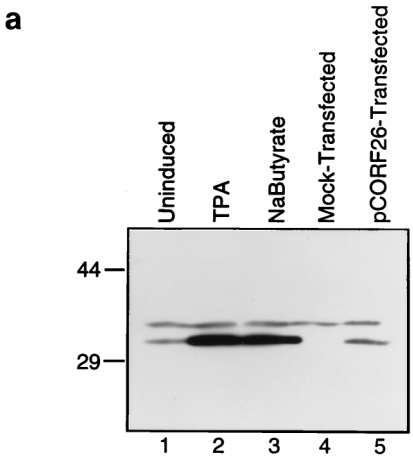
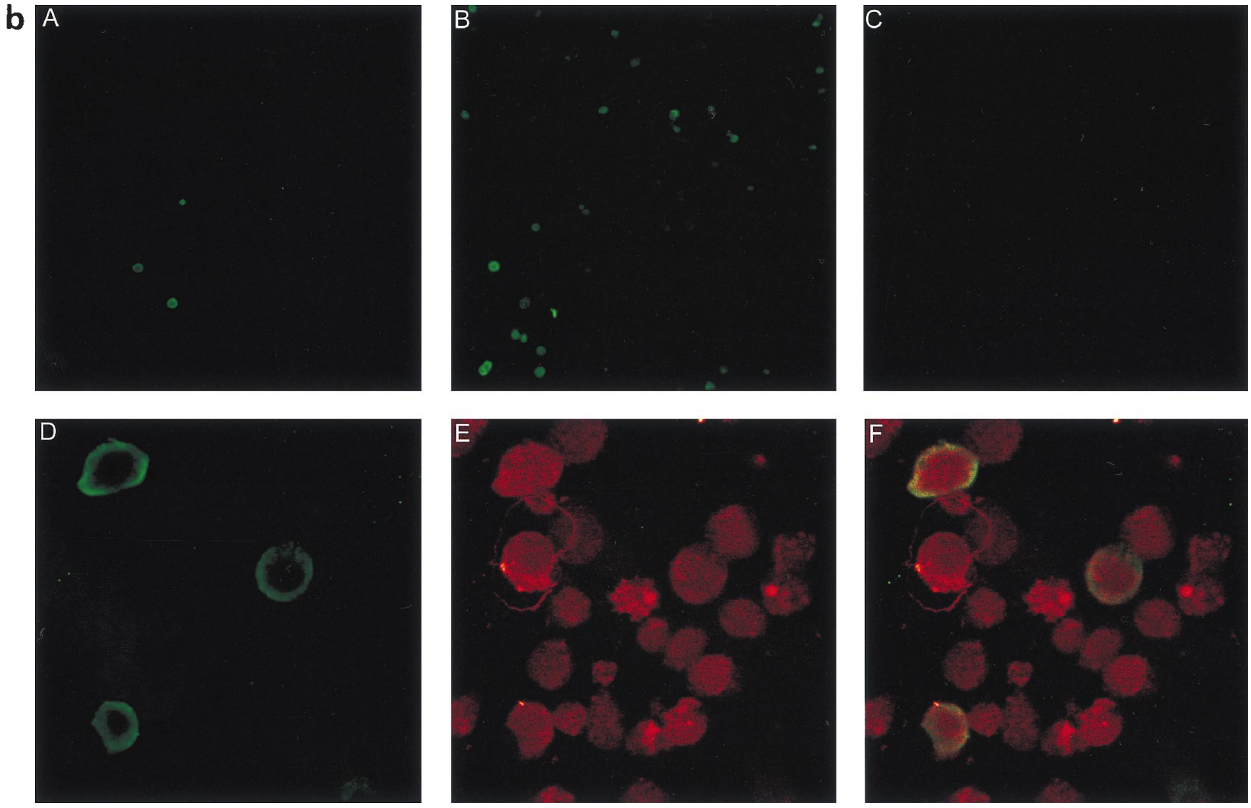


FIG. 4. HHV8 ORF26 expression in BCBL1 cells. (a) Western blot analysis of BCBL1 cells induced with TPA or sodium butyrate for 48 h. Lane 1, uninduced cells; lane 2, cells induced with 20 ng of TPA per ml; lane 3, cells induced with 3 mM sodium butyrate. Lanes 4 and 5 were loaded with 293T cells transfected with 0 or 4 μg of pCORF26 and constituted negative and positive controls, respectively. Cell lysates corresponding to 800 μg of total protein were loaded for each sample. The 32-kDa band present in lanes 1, 2, 3, and 5 (but not in lane 4) corresponds to the ORF26 protein product. Enhanced chemiluminescence was used for detection as described in Materials and Methods. (b) Immunofluorescence analysis of TPA-induced (panels B to F) and uninduced (panel A) BCBL1 cells using affinity-purified HHV8 ORF26 protein antibodies (panels A, B, D, and F) or control affinity-purified rabbit anti-rat IgG (panel C). Panel F corresponds to the superimposition of panels D and E. FITC-labeled goat anti-rabbit IgG was used for detection as described in Materials and Methods. Propidium iodide was included to highlight the nucleus (panels E and F). Magnification, ×82 (panels A to C) and ×603 (panels D to F).



Immunoprecipitation of in vitro-translated HHV8 ORF26 protein. To confirm the presence of antibodies specific for the HHV8 ORF26 protein which do not cross-react with BDLF1, mRNAs encoding the intact ORF26 and BDLF1 proteins were in vitro translated in the presence of [^{35}S]methionine and [^{35}S]cysteine followed by immunoprecipitation analysis. The molecular masses of the resulting proteins were determined to be approximately 32 kDa (HHV8 ORF26 protein) and 33 kDa (BDLF1) (Fig. 2, lanes 9 and 10). Crude anti-HHV8 ORF26 protein antisera as well as affinity-purified antibodies immunoprecipitated the HHV8 ORF26 protein (Fig. 2, lanes 1 to 3) but not BDLF1 (Fig. 2, lanes 4 to 6), confirming the lack of cross-reactivity.

Transient expression of HHV8 ORF26 in 293T cells. To further characterize the affinity-purified anti-HHV8 ORF26protein antiserum, we analyzed 293T cells that transiently expressed HHV8 ORF26. A 32-kDa band was present in the lanes corresponding to the HHV8 ORF26-transfected cells but not in the mock-transfected cells (Fig. 3A, lanes 1 to 5), confirming the molecular weight observed in the in vitro translation experiments (Fig. 2, lane 9). We also used immunofluorescence to analyze the transiently transfected 293T cells for expression of HHV8 ORF26. When affinity-purified anti-HHV8 ORF26 protein antibodies were used, the fluorescence signal was distributed throughout the cytoplasm of the HHV8 ORF26-transfected cells (Fig. 3B). In contrast, affinity-purified rabbit anti-rat IgG used as a control demonstrated no fluorescence (Fig. 3B). No nuclear fluorescence was observed in any sample. These results validate the use of the affinity-purified anti-HHV8 ORF26 protein antibodies for the detection of

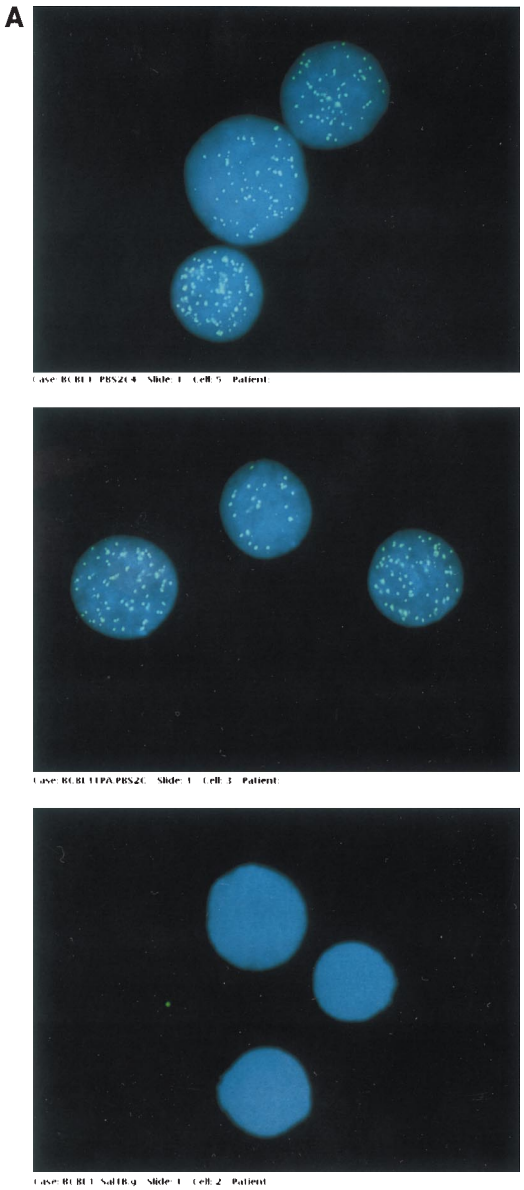


FIG. 5. Transcriptional activation of HHV8. (A) FISH analysis. BCBL1 cells were treated with TPA for 48 h and were hybridized to an HHV8 ORF26-specific probe (middle panel) or a probe specific for the EBV *Sal*IB fragment (bottom panel). Uninduced BCBL1 cells hybridized to the HHV8 ORF26-specific probe are shown on the top panel. (B) Southern blot analysis of HHV8 DNA. DNA from uninduced BCBL1 cells (lane 1) and BCBL1 cells induced with sodium butyrate (lane 2) or TPA (lane 3) was probed with a random-labeled fragment containing the HHV8 ORF26 DNA (as described in Materials and Methods). (C) Northern blot analysis. Total RNA from uninduced BCBL1 cells (lane 1) and BCBL1 cells induced with sodium butyrate (lane 2) or TPA (lane 3) was analyzed by Northern blotting. Virus-specific transcripts were detected as indicated for the Southern blot with an ORF26-specific probe.

HHV8 ORF26 protein by Western blot and immunofluorescence analyses.

HHV8 ORF26 expression in BCBL1 cells. Because no KS cell lines that persistently maintain the HHV8 viral genome are available currently, we used the BCBL1 cell line (27) to study HHV8 ORF26 expression. Using previously published primers in PCR analysis (25), we confirmed the presence of HHV8 DNA in BCBL1 cells (data not shown). In Western blot analysis, affinity-purified anti-HHV8 ORF26 protein antibodies recognized a 32-kDa protein in extracts from BCBL1 cells (Fig. 4a, lane 1). Expression of the ORF26 protein of HHV8 was significantly induced after treatment for 48 h with either TPA or sodium butyrate (Fig. 4a, lanes 1 to 3). This result was confirmed by indirect immunofluorescence analysis. As shown in Fig. 4b (panels A to C), the number of fluorescent cells increased after TPA induction from approximately 1 in every 50 cells to about 1 in every 10 cells. The cytoplasmic distribution of the fluorescence signal observed in both induced and uninduced BCBL1 cells (Fig. 4b, panels D to F) confirmed the cytoplasmic distribution of HHV8 ORF26 protein observed in the transfection experiments described above (Fig. 3B).

HHV8 ORF26 protein expression correlates with the induction of HHV8-specific mRNA. To investigate the level at which TPA treatment increased the expression of the HHV8 ORF26 protein, we performed FISH experiments as well as Southern and Northern blot analyses on BCBL1 cells before and after TPA induction. FISH analysis using DNA probes containing ORF26 confirmed that all of the BCBL1 cells contained HHV8 viral DNA, which on average ranged between 25 and 35 copies per cell (Fig. 5A). Interestingly, under our experimental conditions there was no significant difference in the amount of viral DNA in the TPA-induced and uninduced cells. Control experiments using the *Sal*IB fragment of the EBV genome as a probe demonstrate the absence of EBV in these cells.

To confirm the results from the FISH analysis, a random-labeled 971-bp fragment from HHV8 ORF26 was hybridized to DNA from TPA- or sodium butyrate-induced BCBL1 cells. In agreement with the FISH data, the Southern blot analysis revealed that viral DNA levels increased less than twofold after treatment with TPA (Fig. 5B). In contrast, Northern blot analysis of total RNA with the same probe revealed a significant induction of virus-specific RNAs. A complex pattern of RNA transcripts was observed (Fig. 5C). RNA species of approximately 12, 6.3, 4.1, and 2.1 kb were induced 17-, 7.8-, 1.7-, and 8-fold, respectively, after treatment with sodium butyrate and were induced 26-, 26.3-, 1.9-, and 14-fold, respectively, after treatment with TPA. Transcripts of the same size were observed when poly(A)$^+$-enriched RNA was used for the Northern blot analysis (data not shown). In addition, the three major bands of 12, 6.3, and 2.1 kb hybridized to a strand-specific probe, indicating the same transcriptional orientation as that of ORF26 of HHV8 (data not shown). The pattern of transcription observed highlights the complexity of HHV8 gene expression and confirms the integrity of the preparations of TPA and sodium butyrate used for induction experiments.

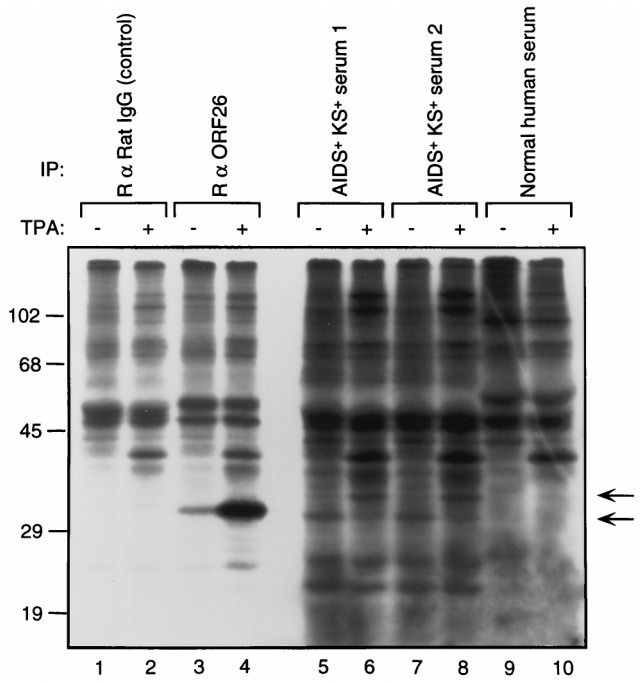


FIG. 6. Immunoprecipitation of HHV8 ORF26 protein from metabolically labeled BCBL1 cells. TPA-treated and untreated BCBL1 cells were metabolically labeled for 3 h in the presence of [^{35}S]methionine and [^{35}S]cysteine. Cell extracts were incubated with rabbit anti-rat IgG (lanes 1 and 2), rabbit anti-HHV8 ORF26 protein antibodies (lanes 3 and 4), AIDS-positive, KS-positive human serum 1 (lanes 5 and 6), AIDS-positive, KS-positive human serum 2 (lanes 7 and 8), or human serum from healthy persons (lanes 9 and 10). Immunoprecipitates were resolved on SDS–12% polyacrylamide gels. The HHV8 ORF26 protein levels were induced 16-fold as determined by phosphorimaging (lanes 3 and 4, lower arrow). The HHV8 ORF26 protein was not present in any of the samples immunoprecipitated with the human sera. However, a distinct band of approximately 34 kDa was immunoprecipitated by the sera from KS patients (upper arrow) and not by the control human sera.

Analysis of antibodies to the HHV8 ORF26 protein in sera from two HIV-infected KS patients. Because ORF26 from HHV8 is expressed at a low level in BCBL1 cultures and at a higher level in induced BCBL1 cells, we tested for the presence of HHV8 ORF26-specific antibodies in the sera from two HIV-positive KS patients. For this purpose, control (uninduced) or TPA-treated (induced) cells were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine. Immunoprecipitation of TPA-induced and uninduced labeled cell extracts with HHV8 ORF26 protein-specific antibodies resulted in a 16-fold induction in the levels of HHV8 ORF26 protein (Fig. 6, lower arrow). This band was absent in both samples immunoprecipitated with sera from KS patients and in the sample immunoprecipitated with control human sera from healthy persons. However, a distinct band of approximately 34 kDa was specifically immunoprecipitated from the TPA-induced BCBL1 cells by both of the KS serum samples (Fig. 6, upper arrow). These results suggest that not all HIV-infected KS patients develop antibodies specific for the protein product of HHV8 ORF26. However, as illustrated by the 34-kDa protein, other yet-to-be-identified proteins induce a detectable humoral response and might serve as markers for infection with HHV8.

DISCUSSION

Since the discovery of DNA sequences corresponding to HHV8 ORF26 in KS DNA (7), evidence regarding the expression of the protein has not been presented. Because KS cell lines that persistently maintain the HHV8 viral genome are currently unavailable, we used the BCBL1 cell line (16, 27, 28) to study HHV8 ORF26 gene expression. Hoping to ultimately identify viral antigens that can be used for epidemiology studies, other groups have used BCBL1 and similar cell lines to screen human sera for antibodies specific to HHV8 proteins (13, 16, 19). The use of recombinant HHV8 proteins to screen human sera is likely to also be useful for these epidemiological studies. However, prior elucidation of the expression patterns (e.g., during latency versus the lytic stage) of the different antigenic candidates is likely to be advantageous.

We generated HHV8 ORF26 protein antibodies by immunizing rabbits with a fusion protein, MBP-ORF26. A second fusion protein, GST-ORF26, was used to demonstrate the presence of antibodies against HHV8 ORF26 protein. Further confirmation was the immunoprecipitation of in vitro-translated HHV8 ORF26 protein with this serum. BDLF1, a capsid protein of EBV, also a gammaherpesvirus which is widely distributed among the human population, has 39% amino acid identity to HHV8 ORF26 protein (7). To validate the anti-HHV8 ORF26 protein antibodies we performed Western blot and immunoprecipitation analyses of BDLF1. In both cases, the rabbit antibodies failed to recognize BDLF1, demonstrating their specificity for HHV8 ORF26 protein and the absence of cross-reactive antibodies against BDLF1.

Transient transfection experiments using 293T cells provided evidence of the usefulness of affinity-purified rabbit antibodies to study the expression of HHV8 ORF26 in the context of a human cell line by both Western blot and immunofluorescence analyses. These experiments allowed us not only to confirm expression of a 32-kDa protein but also to detect the protein's cytoplasmic distribution.

BCBL1 cells were found to express HHV8 ORF26 at high levels upon being induced by TPA or sodium butyrate. Immunofluorescence analysis of TPA- or sodium butyrate-induced cells revealed a fivefold increase in the number of cells showing the cytoplasmic pattern of fluorescence previously observed in the transient transfection experiments. Even though every BCBL1 cell contained HHV8 DNA sequences, only approximately 1 in 50 expressed detectable levels of HHV8 ORF26 protein as determined by immunofluorescence analysis. This is consistent with the previous observation that few cells in the culture spontaneously enter the lytic cycle (27). Even after induction, only about 1 in 10 cells was positive for HHV8 ORF26 protein, suggesting high levels of expression in a few cells. These results highlight the complexity of HHV8 gene expression and suggest a pattern of regulation similar to that of EBV. However, regardless of the induction state, on the basis of the FISH analysis, every BCBL1 cell contained an average of 30 copies of the HHV8 genome. Under our experimental conditions, only small increases in viral DNA levels were observed by Southern blot analysis. At this point, it is unclear why we observed a reduced level of viral DNA induction following TPA treatment compared to the results obtained by other investigators (27, 28). However, it is possible that this difference is due to the use of later passages of the BCBL1 cell line which might not respond as well to the inducers or to differences in reagents used.

The increased amount of ORF26 protein did not correlate with an increase in viral DNA, suggesting a transcriptional effect. Indeed, Northern blot analysis of both total and poly(A)$^+$-selected RNA from induced BCBL1 cells with double-stranded DNA or strand-specific RNA probes demonstrated a complex pattern of transcription in which multiple messages overlap ORF26 in the same transcriptional orienta-

tion. Some of these messages were present at low levels in uninduced cells, consistent with there being a few cells in the lytic stage of the viral cycle (27). Other messages were only found in induced cells. Some of these induced transcripts are likely to represent lytic genes. Further analysis will be necessary to fully elucidate the patterns of transcription of this region of HHV8 to determine the identity of the expressed genes and to identify the transcriptional regulatory elements responsive to TPA and butyrate.

The analysis of immunoprecipitates from induced and uninduced BCBL1 cells with sera from two KS patients failed to show a band corresponding to the ORF26 protein of HHV8. These results suggest a possible absence of antibodies specific for HHV8 ORF26 protein in some KS patients. This could be due to low levels of expression of ORF26 in KS or to a lack of specific immune response. Even though only serum samples from two patients with KS lesions were tested, at this point the ORF26 protein of HHV8 does not appear to be a good candidate for screening human sera for the presence of HHV8-specific antibodies. However, the presence of a 34-kDa immunoprecipitable band recognized by the KS sera and not by the control sera suggests that alternative, yet-to-be-identified proteins might serve as markers of exposure to HHV8. However, further analysis of larger numbers of serum samples will be needed to confirm the possible usefulness of this marker.

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