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Cyclic AMP-Responsive Element-Dependent Activation of Epstein-Barr Virus Zebra Promoter by Human Herpesvirus 6

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We have recently shown that infection of Epstein-Barr virus (EBV) genome-positive B cells by human herpesvirus 6 (HHV-6) results in the expression of the immediate-early EBV Zebra gene, followed by virus replication (L. Flamand, I. Stefanescu, D. V. Ablashi, and J. Menezes, J. Virol. 67:6768–6777, 1993). Here we show that HHV-6 upregulates Zebra gene transcription through a cyclic AMP-responsive element (CRE) located within the Zebra promoter (Zp). Using human B- or T-cell lines transfected with ZpCat reporter gene constructs, we demonstrate that a region designated the ZII domain of Zp is the target of HHV-6 transactivation. Mutation of the consensus AP-1/CRE site within ZII abolished the inducibility of Zp by HHV-6, whereas positioning of the ZII domain upstream of the β-globin minimal promoter conferred responsiveness following HHV-6 infection. Binding of transcription factors to ZII were not induced by HHV-6 or tetradecanoyl phorbol acetate treatment. Binding of these factors to ZII was prevented by oligonucleotides containing CRE but not by AP-1 consensus sequences. Antibodies against CRE-binding (CREB) protein but not against c-Fos or c-Jun were able to supershift the DNA-protein complex, identifying the nature of the transcription factor which binds to ZII as a member of the CREB family of proteins. Finally, transfection of CREB protein and protein kinase A expression vectors were found to activate Zp in Jurkat cells, suggesting that phosphorylated form of CREB protein can play a determining role in the EBV reactivation process.

Epstein-Barr virus (EBV) and human herpesvirus 6 (HHV-6) are pathogenic herpesviruses that are distributed worldwide. EBV infection of human B lymphocytes results in the immortalization of these cells, with the virus remaining in a latent state, expressing a minimum of six nuclear proteins (EBNAs 1, 2, 3A, 3B, and 3C and EBNA LP) and three membrane polypeptides (LMP1, LMP2A, and LMP2B) (for a review, see reference 12). EBV latency can be disrupted by the unique presence of the 38-kDa immediate-early nucleophosphoprotein Zebra (6, 11, 51). Zebra has amino acid regions partially homologous to those found in the product of the cellular proto-oncogene c-*fos* and can transactivate various EBV promoters through DNA binding of AP-1 and cyclic AMP-responsive element (CRE) consensus sequences (5, 35, 36). The Zebra gene (also referred to as the BZLF1 gene) is under the control of a promoter (Zp) containing elements responsive to phorbol esters and anti-immunoglobulin G (IgG) (17, 49). Maximal transcription from this promoter is rapidly observed, within 2 h, and occurs in the presence of protein inhibitors such as cycloheximide and anisomycin, although much less efficiently. Furthermore, Flemington and Speck (19) have described an autoregulatory mechanism of Zebra expression. First, an external stimulus such as tetradecanoyl phorbol acetate (TPA) leads to low-level transcription of the BZLF1 gene. The translated product Zebra binds to a region within Zp (domains ZIIIA and ZIIIB) located between −134 and −104 from the transcription start site and transactivates its own promoter, leading to high levels of Zebra mRNA and protein. Zebra can transactivate various other promoters and is responsible for initiating the cascade of events leading to the complete EBV replicative cycle.

HHV-6 has the ability to infect various cell types, most of which are constituents of the immune system (1, 38). Upon infection of T lymphocytes, HHV-6 can also interact with other viruses, such as human immunodeficiency virus type 1, and transactivate heterologous viral promoters (15, 27). In a previous study (16), we demonstrated that infection of EBV genome-positive human B cells by HHV-6 results in activation of the full EBV replicative cycle. The present study was aimed at defining the molecular mechanisms by which EBV is reactivated following superinfection by HHV-6. Since Zebra activation is a key step for the switch from latency to the lytic cycle, we have focused our study on Zebra activation by HHV-6. Our results indicate that Zp is strongly activated following infection of B and T cells with HHV-6. The element responsive to HHV-6 infection was targeted within the ZII domain of Zp, a region containing AP-1/cyclic AMP-responsive element (CRE) consensus sequence, to which binding of CRE-binding (CREB) or CREB-like proteins is observed, leading to Zp activation.

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MATERIALS AND METHODS

Cell lines and culture conditions. The cell lines Raji, HSB-2, and Jurkat were obtained from the American Type Culture Collection, Rockville, Md. Raji is an EBV genome-positive human B-cell line, HSB-2 is an immature human T-cell line, and Jurkat has a fully mature T-lymphocyte phenotype. All cell lines were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 50 U of penicillin per ml, 50 mg of streptomycin per ml, 30 mg of gentamicin per ml, and 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer.

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Virus production. HHV-6 (GS strain) was propagated in HSB-2 cells as described previously (16). The HHV-6 titer, expressed as the 50% tissue culture infective dose, was determined by scoring the number of HSB-2 cells exhibiting cytopathic effect. The virus stock had a titer of 10^6 50% infective doses per ml. The mock-infected control was prepared from uninfected HSB-2 culture supernatant.

Infection of cell lines. Cells (10^7) were pelleted and either infected with HHV-6 (10^6 50% tissue culture infective doses), mock infected, or treated with UV-irradiated or heat-inactivated HHV-6 for 2 h at 37°C; subsequently, they were resuspended in 10 ml of culture medium. Virus inactivation was performed as described previously (16). In some experiments, cells were treated with phosphonoacetic acid (PAA; an inhibitor of viral DNA polymerase activity) for 1 h prior to infection. The cells were harvested at various time points, washed with phosphate-buffered saline (PBS; pH 7.4) and processed for RNA isolation, fluorescence microscopy, chloramphenicol acetyltransferase (CAT) assays, and gel mobility retardation assays.

RNA isolation and Northern (RNA) blot analysis. Total RNA was isolated by guanidium isothyocyanate-phenol-chloroform extraction as described previously (9). Total RNA was separated by electrophoresis through a 1% agarose gel containing formaldehyde. RNA was transferred onto nylon membranes and UV cross-linked before prehybridization in 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7])–50% formamide–5× Denhardt's solution–100 μg of denatured salmon sperm DNA per ml–0.5% sodium dodecyl sulfate (SDS) at 42°C for 2 h. Membranes were probed for BZLF1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA with ^{32}P-labelled probes. BZLF1 probe corresponds to the *Bam*HI Z fragment of EBV genome, and GAPDH probe corresponds to the cDNA of the gene. Following overnight incubation at 42°C, membranes were washed twice at room temperature in 6× SSPE–0.5% SDS and twice at 42°C in 1× SSPE–0.1% SDS before being exposed to film at −80°C.

Immunofluorescence assays. PBS-washed Raji cells were processed for immunofluorescence as described previously (16). Following 1 h of incubation with the BZ.1 anti-Zebra (a gift from L. S. Young and M. Rowe) or 2D6 anti–HHV-6 gp105/82 (gift from N. Balachandran) monoclonal antibodies, slides were washed in PBS, and the fluorescein-conjugated goat anti-mouse IgG secondary antibody (Ortho Diagnostics, Raritan, N.J.) was added for 1 h. The slides were then washed, mounted, and examined in a Zeiss Axioskop fluorescence microscopy. The percentage of positive cells was calculated after counting at least 200 cells. Statistical significance was determined by the χ^2-test. A P value of <0.05 was considered significant.

CAT assays. Cells (10^7) were transfected by the DEAE-dextran procedure. Briefly, the cells were washed in TD (20 mM Tris-HCl [pH 7.4], 137 mM NaCl, 5 mM KCl, 0.37 mM $Na_2HPO_4 \cdot H_2O$, 50 mM $MgCl_2$, 90 mM $CaCl_2$). Cell pellets were resuspended in 1 ml of TD containing 500 μg of DEAE-dextran (Pharmacia, Dorval, Canada) and plasmid DNA (1 to 10 μg) and then incubated at room temperature for 20 min, after which 10 ml of medium containing 100 μM chloroquine (Sigma, St. Louis, Mo.) was added. Following a 45-min incubation at 37°C, the cells were pelleted and resuspended in fresh culture medium. The cells were allowed to recover for 24 h before being infected with HHV-6 as described above. Infection was allowed to proceed for 24 to 72 h before CAT activity was determined. The fold induction in CAT activity was determined by scanning densitometry of the exposed films or by liquid scintillation counting of acetylated chloramphenicol. All CAT constructs used were generously given by E. Flemington, Dana-Farber Cancer Institute, Boston, Mass.; these include −221Zpcat, −221 MII Zpcat, −159Zpcat, −129Zpcat, −105Zpcat, −105 MII Zpcat, −86Zpcat, −65Zpcat, BG-cat, and ZII BG-cat (18).

Luciferase assays. For greater sensitivity, the −86ZpCat and −65ZpCat reporter vectors were converted to −86Zpluc and −65Zpluc, respectively, by deleting the CAT gene and replacing it with the luciferase gene. These constructs were cotransfected with expression vectors encoding the catalytic subunit of protein kinase A (PKA) (provided by S. McKnight [46]) and GAL4-CREB proteins (provided by R. A. Maurer [50]). At 72 h posttransfection, cells were washed, counted, and lysed for 15 min in 500 μl of buffer (25 mM Tris [pH 7.8], 2 mM EDTA, 2 mM dithiothreitol, 10% glycerol, 1% Triton X-100). After brief centrifugation, 20-μl volumes of each sample, in triplicate, were tested for luciferase activity on a Biolumat LB9500C luminometer (Berthold Analytical Instruments Inc.).

Electrophoretic mobility shift assay. Jurkat cells (2×10^7 per sample) were treated with either mock infection fluid, HHV-6 (2×10^6 $TCID_{50}$), or TPA (50 ng/ml) for periods varying from 1 to 8 h, after which nuclear extracts were obtained essentially as described by Dignam et al. (14). Briefly, cells were washed in ice-cold PBS and resuspended in hypotonic buffer (buffer A of Dignam et al. [14]). Cytoplasmic membranes were ruptured by addition of 0.5% Nonidet P-40 (final concentration). Nuclei were pelleted, washed, and resuspended in 2 volumes of buffer C (14). Extracts were incubated at 4°C for 1 h, after which the samples were centrifuged at 100,000 × g for 30 min. Supernatants containing nuclear proteins were diluted with an equal volume of buffer D, and the protein concentration was determined by the bicinchoninic acid protein assay (Pierce, Rockford, Ill.) before the samples were frozen at −80°C. Typical binding reactions were carried out as follows. Protein (2 μg) was incubated with 3 μg of poly(dI-dC) poly(dI-dC) (Pharmacia), with or without unlabelled ZII (40 ng) (5′-CCCAAACCATGACATCACAGAG-3′), CRE (5′-GATCCGGCTGACGTCATCAGCTA-3′), or AP-1 (5′-CGGTTGATGAGTCAGCCGGAA-3′) double-stranded oligonucleotides for 15 min on ice. ^{32}P-end-labelled ZII, CRE, or AP-1 oligonucleotides (0.2 ng) were added, and the mixtures were left at room temperature for 30 min before being loaded onto a 5% nondenaturing Tris-glycine polyacrylamide gel. Migration was carried out at 60 mA, and the gels were dried and exposed to X-ray films.

Supershift experiments were performed essentially as described above. Following the 30-min incubation, antibodies (1 μl) against CREB protein, binding to residues 92 to 124 in the P-box region of CREB 327 (42, 52; a gift from J. Habener, Massachusetts General Hospital, Boston, Mass.), or antibodies directed against c-*fos* or c-*jun* (Oncogene Science) were mixed with samples for an additional 30 min before electrophoresis.

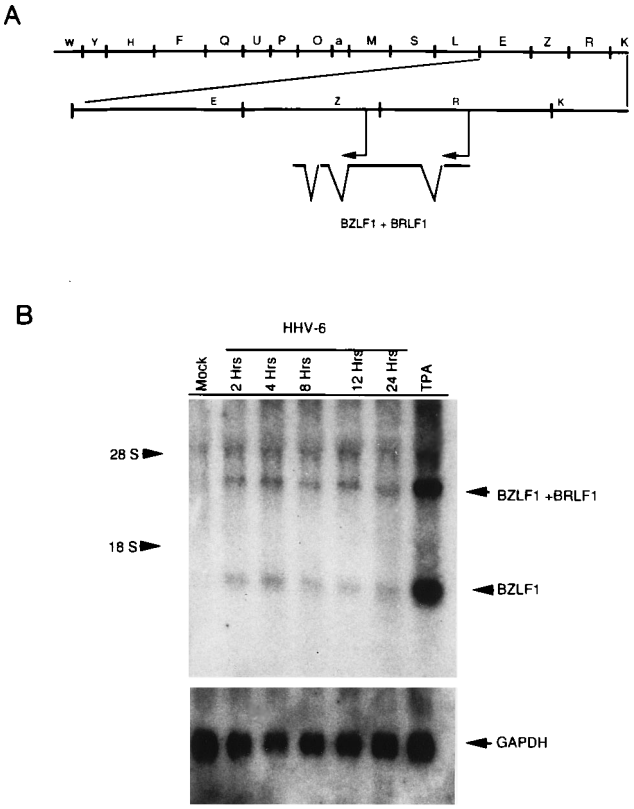

FIG. 1. Kinetics of BZLF1 RNA expression following infection of Raji cells with HHV-6. Raji cells (10^7) were either treated with mock infection fluid, infected with HHV-6, or stimulated with TPA for various periods. Total RNA was isolated and separated by electrophoresis in a formaldehyde-containing agarose gel. Following transfer, RNA was probed for BZLF1 mRNA by using the ^{32}P-labelled *Bam*HI Z genomic fragment of EBV and for the housekeeping GAPDH mRNA. (A) *Bam*HI restriction map of the EBV genome along with the two sites (from Z and R promoters) of initiation of the BZLF1 mRNA. (B) Expression of BZLF1, BZLF1 plus BRLF1, and GAPDH mRNAs after infection by HHV-6.

RESULTS

Kinetics of BZLF1 mRNA expression following infection of Raji cells with HHV-6. The kinetics of BZLF1 gene expression was examined following infection of Raji cells with HHV-6. BZLF1 is an immediate-early gene which is expressed rapidly following stimulation of EBV genome-positive B cells. RNA was extracted beginning at 2 h after HHV-6 infection and up to 24 h. Results (Fig. 1) indicate that as soon as 2 h postinfection, an increase in the BZLF1 mRNA level is observed. The probe used, *Bam*HI Z fragment of EBV genome, recognizes both the monocistronic BZLF1 and bicistronic BZLF1-BRLF1 mRNAs, transcribed from the Z and R promoters, respectively (Fig. 1A). Increased expression of BZLF1 mRNA was also observed at 4 h postinfection, after which time a slow decrease in BZLF1 expres-

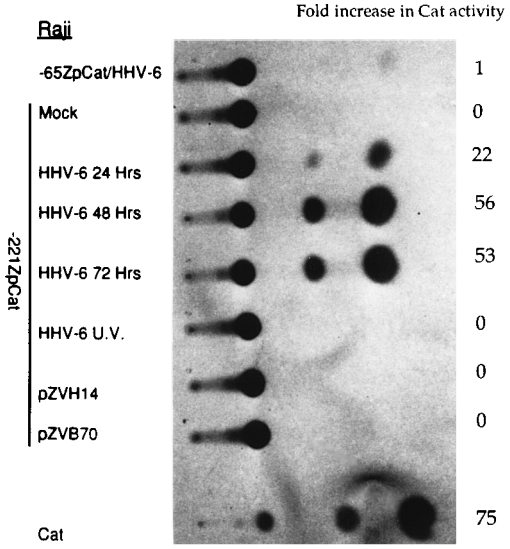

FIG. 2. Zebra promoter activity in Raji cells following infection with HHV-6. Raji cells were transfected with Zebra promoter (−221Zpcat and −65Zpcat) constructs, as described in Materials and Methods. Transfected cells were infected with live HHV-6 or UV-irradiated HHV-6 for various periods, after which CAT activity was determined. Raji cells were also cotransfected with −221Zpcat and pZVB70 or pZVH14. Purified CAT enzyme was included as a positive control. Fold increase in CAT activity was determined by scanning densitometry of the autoradiographs. Data are representative of three independent experiments.

sion was noted. TPA-stimulated Raji cells (2 h) were used as a positive control. Expression of the GAPDH housekeeping gene is also shown in Fig. 1B.

Zebra protein expression following infection of Raji cells with HHV-6. To ascertain that HHV-6-induced BZLF1 gene expression was correlated with an increase in Zebra protein, HHV-6-infected Raji cells were processed at 24 h for immunofluorescence and analyzed for Zebra expression. On average, 6 to 8% of Raji cells were positive for Zebra antigen following infection with HHV-6 (data not shown). This represents a greater than 10-fold increase in Zebra-positive cells compared with mock-infected ones. Approximately 25% of Raji cells were found to react with the BZ.1 anti-Zebra monoclonal antibody following TPA treatment (positive control). HHV-6 infection of Raji cells was confirmed by detecting gp105/82 of HHV-6 with the 2D6 monoclonal antibody. Strong cytoplasmic staining was seen in HHV-6-infected cells, of which between 20 and 30% were positive for gp105/82 of HHV-6.

Zp activation following infection with HHV-6. We have shown that HHV-6 infection of Raji cells results in the induction of BZLF1 mRNA and Zebra protein expression. To investigate whether HHV-6 had any effect on Zp of EBV, Raji cells were transfected with two CAT constructs, one containing the full Zp (−221 to +12 from the transcription start site) (−221 Zpcat) and the other containing 65 nucleotides upstream of the transcription initiation site (−65Zpcat). Results indicate (Fig. 2) that HHV-6 activates the full Zp (−221Zpcat) whereas it has no effect on the 5′-deleted −65Zpcat. Kinetics of infection indicate that maximal Zp activation (56-fold) occurs within 48 h. Furthermore, we tested whether viral infectivity was required to observe Zp activation. UV-irradiated HHV-6 failed to stimulate Zp to any extent, indicating the need for infectious virus. Similar results were obtained with heat-inactivated virus (data not shown). Cotransfection of −221Zpcat with pZVB70 or pZVH14 genomic segments of HHV-6, which are known to transactivate various heterologous promoters such as the human immunodeficiency virus long terminal repeat, failed to stimulate Zp of EBV. In addition, five other HHV-6 genomic segments, some of which encode immediate-early proteins, known to transactivate the human immunodeficiency virus long terminal repeat, were tested and found not to activate Zp to any extent. These were pEPLF3, pIEGP2-3′, pIEG1-2, pBCLF0/1 (obtained from J. Nicholas), and B115 (obtained from A. Razzaque). Purified CAT enzyme was included as a positive control of assay reactions.

Effect of the viral DNA polymerase inhibitor PAA on Zp activation by HHV-6. All previous transfection experiments were performed with Raji cells. Since it is known that Zebra can transactivate its own promoter (19), all subsequent transfection experiments were performed with the EBV-negative Jurkat T-cell line. Our results (Fig. 3) indicate that transfection of −221Zpcat in these cells, followed by infection by HHV-6, leads to levels of Zp activation similar to those observed in B cells. Second, knowing that HHV-6 infectivity is required to activate Zp of EBV, we tested the effect of PAA on the activation of Zp by HHV-6. The results (Fig. 3) suggest that Zp is activated by HHV-6 in both the presence and absence of PAA. Different concentrations of PAA had no effect on the ability of HHV-6 to stimulate Zp.

Identification of the Zp region(s) essential for activation by HHV-6. The Zp contains multiple domains, some of which are responsive to TPA-inducible factors while others bind Zebra itself. To determine the minimal promoter required for Zp activation by HHV-6, we have transfected a series of 5′-deleted Zp constructs into T cells and tested them for responsiveness to infection by HHV-6. As shown in Fig. 4, all Zp constructs, with the exception of −65 Zpcat, were activated following infection with HHV-6. There were no differences in CAT activity between the various Zp constructs (from −221 to −86). Transfection of different quantities (1 to 10 μg) of −86Zpcat plasmid into Jurkat cells followed by infection with HHV-6 led to a plateau in promoter activity response with 5 μg of plasmid DNA (data not shown).

Having determined that the minimal promoter responsive to HHV-6 contains 86 nucleotides upstream of the transcription start site, we next studied the potentially important sites within this region. Sequence analysis of these nucleotides suggested

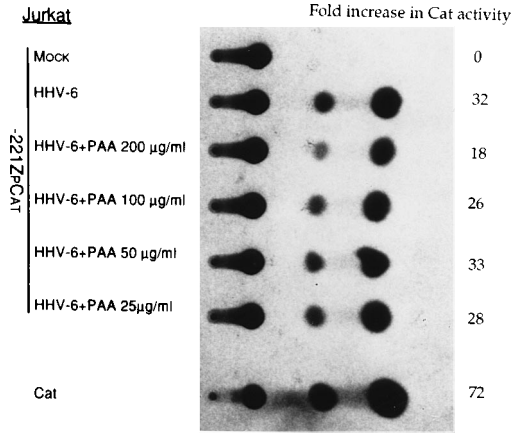

FIG. 3. Effect of PAA on Zebra promoter induction by HHV-6. Jurkat cells were transfected with −221Zpcat plasmids. Transfected cells were incubated in the presence of increasing concentration of PAA for 1 h before infection with HHV-6. After 48 h, cytoplasmic extracts from cells were obtained and analyzed for CAT activity. Similar results were obtained in three separate experiments.

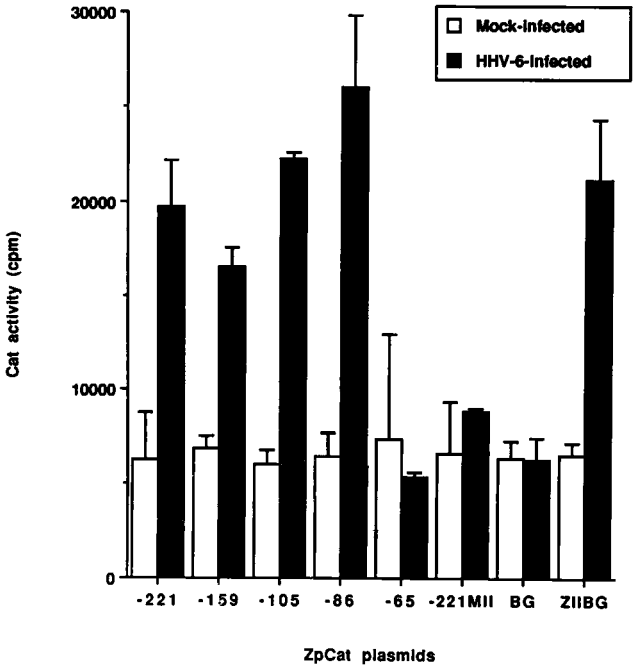

FIG. 4. Identification of the minimal Zebra promoter and localization of the Zp domain essential for EBV activation by HHV-6 infection. Jurkat cells were transfected with a series of 5′-deleted Zpcat constructs (−221 to −65), a mutant form of −221ZpCat termed −221 MII Zpcat, a minimal β-globin promoter (BG), and the BG upstream of which the ZII domain of Zp was cloned (ZIIBG). At 48 h after HHV-6 infection, CAT activity was determined in each sample. Data shown are representative of two experiments.

that there was a putative AP-1 or CRE consensus sequence (TGACATCA) located between −67 and −60. To determine the importance of this sequence, cells were transfected with plasmids containing wild-type and mutated AP-1/CRE sites (ATTCATCA) (−221MIIZpcat). As shown in Fig. 4, wild-type promoter constructs were activated severalfold while promoters containing a mutated AP-1/CRE site could not be activated following infection by HHV-6, suggesting that this region of Zp is essential for responsiveness. Furthermore, cloning of the ZII domain of Zp, containing the CRE/AP-1 sequences, upstream of the minimal BG promoter (ZIIBG) was able to restore responsiveness to levels similar to those found in cells transfected with wild-type Zp following HHV-6 infection. This indicates that ZII by itself is sufficient and does not rely on any *cis*-acting elements found within Zp to promoter responsiveness to HHV-6 infection.

ZII domain of Zp binds noninducible, constitutively expressed, nuclear factors. The presence of a putative AP-1/CRE site essential for activation by HHV-6 led us to study proteins capable of interacting with the ZII domain. Double-stranded oligonucleotides encompassing the AP-1/CRE site found within ZII were used to study the interaction of the DNA-binding protein with ZII and possibly to mediate Zp activation. By electrophoretic mobility shift assay, it was observed that mock- and HHV-6-infected Jurkat cells were expressing nuclear proteins capable of binding to the ZII oligonucleotides (Fig. 5). Two distinct complexes with different electrophoretic mobilities were observed. Protein binding was specific and could be eliminated by preincubation with excess unlabelled ZII oligonucleotides. Kinetic studies of infection did not lead to an increase in binding, and neither did cell treatment with TPA, suggesting that proteins binding to ZII are constitutively expressed and are not induced by HHV-6 or TPA.

Binding of proteins to ZII can be efficiently inhibited by oligonucleotides containing CRE consensus sequences. To characterize further the constitutively expressed factors capable of binding to ZII, we tested the ability of oligonucleotides containing CREB (5′-TGACGTCA-3′) or AP-1-binding (5′-TGAGTCA-3′) sequences to compete for ZII binding. Nuclear extracts from 4-h HHV-6-infected Jurkat cells were found to contain proteins capable of binding ZII (Fig. 6, lane 1). Incubation of extracts in the presence of excess unlabelled ZII (lane 2) or CRE (lane 3) oligonucleotides was found to reduce binding of both complexes to a minimum, whereas AP-1 oligonucleotides did not affect binding of proteins to the more slowly migrating (upper) complex (lane 4). CRE oligonucleotides were at least as efficient as ZII in competing for protein binding. Having determined that CRE oligonucleotides can efficiently compete with ZII for protein binding, we studied proteins within the same extracts capable of binding to CRE. As shown (lane 5), the nuclear extracts contained proteins binding to CRE oligonucleotides. However, with equal quantities of protein, more binding was observed with the CRE oligonucleotides than with those bound to the ZII oligonucleotide (lane 1). The electrophoretic mobility patterns of both complexes bound to CRE were identical to those of the complexes bound to ZII. Incubation of nuclear extracts with excess cold ZII (lane 6) partially inhibited binding to CRE, while complete removal of binding was observed in the presence of excess cold CRE oligonucleotides (lane 7). Less efficient binding to and competition by ZII oligonucleotides can be explained by the consensus sequence of ZII differing by 1 nucle-

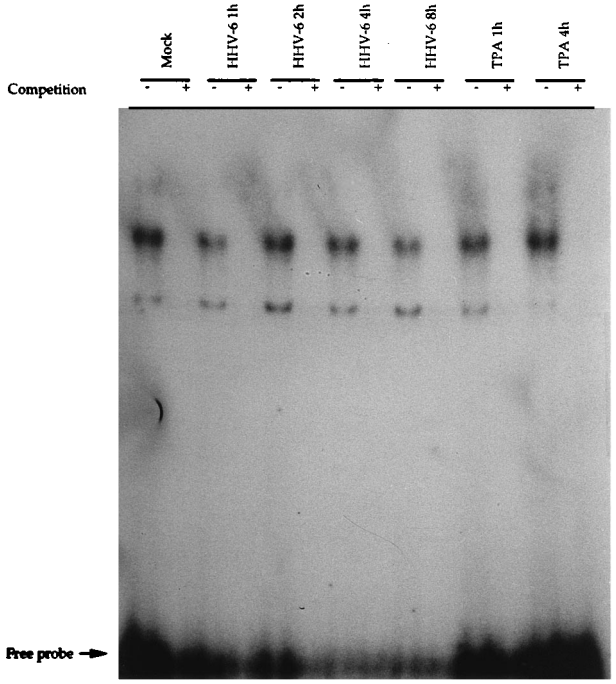

FIG. 5. Noninducible, constitutively expressed nuclear proteins bind AP-1/CRE of ZII. Nuclear extracts (2 μg) from mock-infected, HHV-6-infected, or TPA-treated Jurkat cells, obtained as described in Materials and Methods, were incubated on ice for 15 min in the presence (+) or absence (−) of unlabelled ZII oligonucleotides (40 ng), after which 0.2 ng of ^{32}P-end-labelled ZII oligonucleotides was added for 30 min at room temperature. Samples were loaded on 5% nondenaturing Tris-glycine-EDTA polyacrylamide gels and migrated at 60 mA, after which the gels were dried and exposed to films.

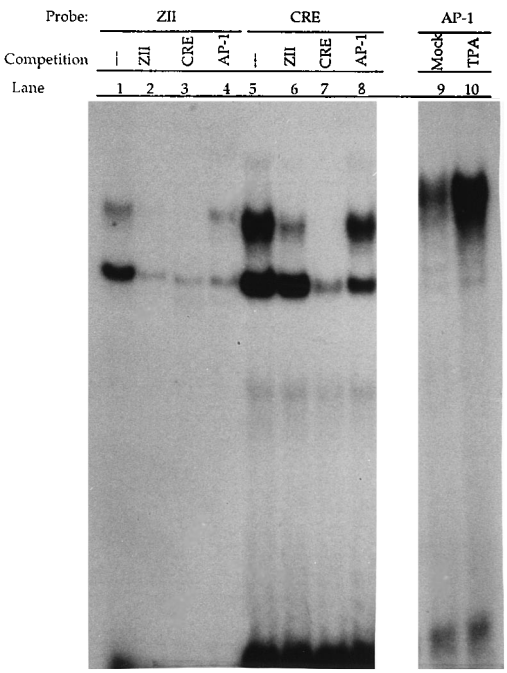

FIG. 6. Competition by CRE but not AP-1 oligonucleotides for protein binding to ZII. Nuclear extracts (2 μg) from 4-h HHV-6-infected Jurkat cells were incubated with 40 ng of unlabelled ZII (lanes 2 and 6), CRE (lanes 3 and 7), or AP-1 (lanes 4 and 8) oligonucleotides for 15 min on ice, after which 0.2 ng of ^{32}P-labelled ZII (lanes 1 to 4) or CRE oligonucleotides (lanes 5 to 8) was added for 30 min at room temperature. Also shown is the binding of proteins to AP-1 oligonucleotides (lanes 9 and 10). Extracts from mock-infected (lane 9) and TPA-treated (lane 10) Jurkat cells were allowed to bind to 0.2 ng of ^{32}P-labelled AP-1 oligonucleotides before electrophoresis, as described in the legend to Fig. 5.

otide from the wild-type CRE sequence. Excess AP-1 oligonucleotides had marginal effects on protein binding to CRE (lane 8). Not all of the faster-migrating complex can be inhibited with cold ZII or CRE oligonucleotides, suggesting that factors other than CREB-like proteins attach to this sequence. Also shown is the binding of nuclear proteins from mock-infected and 4-h TPA-treated Jurkat cells to AP-1 oligonucleotides. Binding to AP-1 is increased by TPA (compare lanes 9 and 10), and the migration pattern of proteins is considerably different from that of those bound to ZII (lane 1) and CRE (lane 5).

Proteins bound to ZII can be supershifted with anti-CREB protein serum. Evidence obtained by electrophoretic mobility shift assay suggests that proteins bound to ZII have identical electrophoretic mobility to those bound to CRE and that their binding can be prevented by preincubation with CRE oligonucleotides. To confirm that complexes bound to ZII were CREB proteins, nuclear extracts from HHV-6-infected Jurkat cells were incubated with ZII oligonucleotides and then with antibodies against c-*fos*, c-*jun*, and CREB protein. By electrophoretic mobility shift assay, it was observed (Fig. 7) that antibodies against c-*fos* or c-*jun* had no effect on the electrophoretic mobility of complexes bound to ZII (compare lane 1 with lanes 2 and 3). However, incubation of extracts with anti-CREB protein serum led to a retardation in the mobility of the two protein complexes (supershift) bound to ZII (lane 4), indicating the interaction of anti-CREB protein antibodies with proteins bound to ZII. The faster-migrating complex was not totally supershifted by CREB protein antiserum, indicating the presence of nonimmunologically reactive proteins bound to ZII.

Phosphorylated CREB protein can efficiently activate the Zebra promoter. The identification of CREB or CREB-like proteins binding Zp through its ZII domain suggests a role for this factor in Zebra gene activation. CREB proteins are members of the cAMP-responsive factor family and rely upon phosphorylation by various kinases, including PKA, Ca^{2+}-calmodulin-dependent kinase, and PKC (21, 40, 50, 53) for transcriptional activity. We next determined the effect of forskolin, a known adenylate cyclase activator, on Zp activation. By itself, forskolin had no effect on −86Zpcat-transfected Jurkat cells (Fig. 8A). However, when forskolin was added together with HHV-6, a significant increase ($P < 0.05$) in activity was observed when compared with cultures treated with HHV-6 only. To further demonstrate that CREB protein can in fact transactivate Zp, Jurkat cells were transfected with −86Zpluc along with vectors expressing the catalytic subunit of PKA and GAL4-CREB fusion protein. As shown in Fig. 8B, transfection of either CREB or PKA expression vectors alone did not result in significant activation of −86Zpluc. However, when the two expression vectors were combined, a highly significant ($P < 0.01$) increase in luciferase activity was registered, suggesting that only phosphorylated CREB protein can efficiently activate Zp. No effect on −65Zpluc was observed under the same conditions (data not shown).

DISCUSSION

Herpesviruses have a complex gene regulation. In the case of EBV, viral expression during latency is limited to nine genes. During the replicative cycle, genes are expressed in a temporal fashion, with the immediate-early genes transcribed first. These gene products then activate early genes, which in turn stimulate transcription of the late genes, completing the cycle. One such immediate-early transactivator is Zebra, encoded by the BZLF1 gene of EBV. Zebra expression in latently infected cells is presumably silenced by negative *cis*-acting regulatory elements (−386 to −434) found within Zp (43). However, upon cell stimulation with TPA or anti-IgG, Zebra gene expression is turned on and the replicative cycle of EBV is initiated (6, 11, 51). Regions of Zp responsive to TPA and anti-Ig activation have been identified (18, 49). However, Zp is minimally activated by TPA in non-EBV-carrying cell lines, suggesting that TPA-responsive elements may not be sufficient to

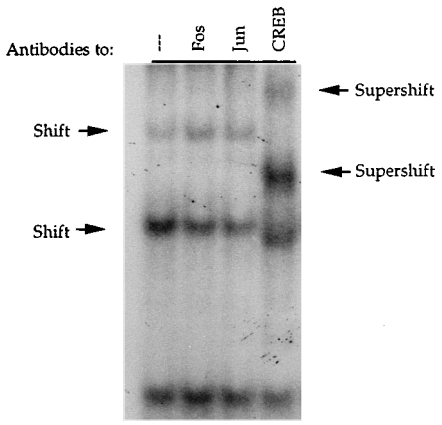

FIG. 7. Proteins bound to ZII can be supershifted with anti-CREB protein serum. Nuclear extracts from 4-h HHV-6-infected Jurkat cells were incubated with ^{32}P-labelled ZII oligonucleotides for 30 min at room temperature. Antibodies against c-Fos, c-Jun, or CREB protein were added to the mixtures, and incubation was prolonged for an additional 30 min. Samples were electrophoresed as described in the legend to Fig. 5.

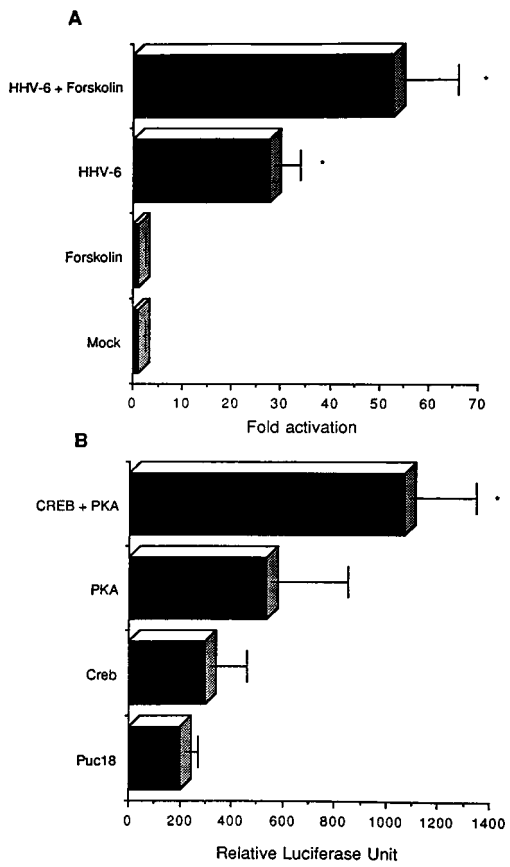

FIG. 8. Enhancement of Zp activation by forskolin and phosphorylated CREB. (A) Jurkat cells were transfected with −86Zpcat reporter construct and infected with HHV-6. The effect of forskolin was then determined by adding 10 μM of this chemical to both uninfected and infected cultures. The enhanced (*, $P < 0.05$) CAT activity shown was determined 48 h postinfection. (B) Jurkat cells were transfected with Zpluc reporter vector (2 μg) with or without CREB (1 μg) and PKA (1 μg) expression vectors. DNA input was normalized with puc18 DNA. At 72 h posttransfection, cells were harvested and tested for luciferase activity as described in Materials and Methods. Results are presented as mean ± standard deviation of relative luciferase units derived from three independent transfections (*, $P < 0.01$).

fully activate Zp in the absence of EBV proteins (15a, 49). Having observed that HHV-6 can efficiently activate Zp in both EBV-carrying B cells and T cells, we sought to identify other responsive elements participating in Zp activation.

Promoter elements responding to cAMP are referred to as CRE (cAMP-responsive elements) (10, 13, 47). Factors binding to CRE sequences (TGACGTCA) such as CREB protein or CRE-modulating (CREM) protein belong to the leucine zipper (b-Zip) class of proteins (20, 22, 23, 26). CREB and CREM proteins share homologies with b-Zip proteins capable of binding to TPA-responsive elements such as the AP-1 transcription complex, composed of homodimerized Jun or of the Fos-Jun heterodimer (4, 8, 32, 48). AP-1 is induced by TPA stimulation and regulates the expression of many genes having TPA-responsive elements in their promoter (3, 34). By contrast, proteins binding to CRE are constitutively expressed in cells and can exert both silencing and stimulating effects. CREM protein prevents gene activation by occupying CRE sites either as a homodimer or as a CREM-CREB protein heterodimer (20). It is thought that upon cAMP induction, phosphorylated CREB dimers override the negative CREM protein influence and promoter gene transcription (20). The EBV Zp contains a DNA sequence (TGACATCA) related to both AP-1 and CRE (18). Such sequence has been shown to bind AP-1 (Jun plus Fos), but transactivation of Zp by Jun in the Ramos B-cell line was not observed (18). Interestingly, a sequence identical to the AP-1/CRE site found in Zp is also present in the c-*jun* promoter (7). This sequence was shown to bind both CREM and CREB proteins, causing downregulation of c-*jun* gene expression. Such inhibition can be reversed by phosphorylation of CREB protein by PKA (31). In the present work, we provide evidence that superinfection of the EBV genome-positive Raji cell line by HHV-6 results in the induction of Zebra mRNA. Moreover, our results indicate that HHV-6 can activate Zp independently of any EBV-encoded proteins. Using 5′-deleted Zp constructs together with mutagenized Zp, we have identified a region between −86 and −65 of Zp essential for activation by HHV-6. This region contains the consensus AP-1/CRE sequences mentioned above. We show that CREB or CREB-like proteins are involved in Zp activation following HHV-6 infection, since (i) mutation of the CRE site within the ZII domain of Zp abolished responsiveness to HHV-6 infection; (ii) noninducible, constitutively expressed factors were found to bind the putative CRE site found in ZII, in agreement with intrinsic biological properties of CREB proteins; (iii) protein binding to the putative CRE site of ZII was prevented by oligonucleotides containing wild-type CRE consensus sequence; (iv) proteins, whether bound to oligonucleotides with wild-type CRE or ZII-CRE, have the same electrophoretic mobility; (v) proteins bound to ZII-CRE can be supershifted by anti-CREB protein antiserum; and (vi) cotransfection of vectors driving the expression of CREB protein and the catalytic subunit of PKA was able to activate Zp. In addition, activation of Zp by HHV-6 was enhanced by forskolin, which indirectly activates PKA through an increase in intracellular cAMP levels. Evidence against the involvement of AP-1 in Zp activation by HHV-6 includes the following: (i) it is not possible to supershift proteins bound to ZII by antibodies against c-*fos* and c-*jun*; (ii) protein complexes bound to ZII have different electrophoretic mobility from those bound to oligonucleotides with AP-1 sequence; (iii) AP-1 oligonucleotides cannot compete efficiently with ZII for binding of proteins; and (iv) TPA treatment of cells does not modulate the binding of proteins to ZII but does increase AP-1 binding.

To our knowledge this is the first report of a CRE sequence-dependent activation of BZLF1 promoter of EBV. Previous reports have identified TRE sequence involvement in EBV reactivation following TPA or anti-IgG stimulation of cells (33, 41). Our approach has the advantage of enabling us to study the activation of Zebra in the absence of EBV proteins which might influence this process. Utilization of cellular transcription factors by viruses for regulation of promoter activity is common. The most thoroughly studied of all recently isolated viruses, human immunodeficiency virus, is known to make use of factors such as NF-κb (24, 44) and SP1 (29) to facilitate its gene transcription. Another retrovirus, the bovine leukemia virus, utilizes CREB proteins in Tax-mediated long terminal repeat transactivation (30). Furthermore, the adenovirus E1A oncoprotein seems to exert its transactivating effect in conjunction with CREB-related proteins (25, 28, 37, 47). The utilization of CREB proteins by HHV-6 to promote gene transcription may represent an evolutionary adaptation of this virus. In fact, an AP-1 site capable of binding both CREB protein and AP-1 transcriptional complexes is found upstream of the putative immediate-early gene locus of HHV-6 (39). Furthermore, it was recently shown that the HHV-6 DNA polymerase promoter can be transactivated after infection by HHV-6

through a CRE consensus sequence (2). CREB protein activation following infection by HHV-6 could therefore lead to enhanced transcription of viral genes, enabling a more efficient and rapid triggering of events during initiation of infection. We are currently working at identifying the nature of the HHV-6 factor(s) involved in CREB protein activation. It is noteworthy, however, that seven HHV-6 genomic segments which were known to transactivate various heterologous promoters and some of which encoded immediate-early protein, were tested during this study and were found to be ineffective in this activation.

On the basis of the present findings, we hypothesize that CREB/CREM protein occupancy of the CRE site within Zp of EBV causes silencing of the BZLF1 gene and favors a latent EBV infection. Upon infection of B lymphocytes by HHV-6, a virus-encoded product leads to CREB protein phosphorylation, activation of Zp, and transcription of the gene. Zebra protein then transactivates various EBV promoters, initiating the cascade of events leading to EBV reactivation. Release of infectious EBV particles can lead to an increase in virus load and an increase in the number of EBV-infected B lymphocytes, which favor the development on EBV-related diseases such as B-cell lymphoproliferation. In immunodeficient individuals, a correlation exists between the degree of EBV reactivation and the incidence of EBV-related lymphomas. Infection by, or reactivation of, HHV-6 in immunocompromised hosts may therefore contribute dually to pathogenesis, i.e., through HHV-6-associated pathologies and through reactivation of EBV.

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