JOURNAL OF VIROLOGY, Nov. 1998, p. 8797–8805 Vol. 72, No. 11
0022-538X/98/$04.00+0


# CD4 Promoter Transactivation by Human Herpesvirus 6

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Received 26 February 1998/Accepted 4 August 1998

**The observation that human herpesvirus 6 (HHV-6) can induce CD4 gene transcription and expression in CD4$^-$ cells was reported several years ago (P. Lusso, A. De Maria, M. Malnati, F. Lori, S. E. DeRocco, M. Baseler, and R. C. Gallo, Nature 349:533–535, 1991) and subsequently confirmed (P. Lusso, M. S. Malnati, A. Garzino-Demo, R. W. Crowley, E. O. Long, and R. C. Gallo, Nature 362:458–462, 1993; G. Furlini, M. Vignoli, E. Ramazzotti, M. C. Re, G. Visani, and M. LaPlaca, Blood 87:4737–4745, 1996). Our objective was to identify the mechanisms underlying such phenomena. Using reporter gene constructs driven by the CD4 promoter, we report that HHV-6 can efficiently transactivate such genetic elements. Activation of the CD4 promoter occurs in the presence of the viral DNA polymerase inhibitor phosphonoformic acid, which limits expression to the immediate-early and early classes of viral genes. Using deletion mutants and specific CD4 promoter mutants, we identified an ATF/CRE binding site located at nucleotides −67 to −60 upstream of the CD4 gene transcription start site that is important for HHV-6 transactivation. The ATF/CRE site is also essential for CD4 promoter activation by forskolin, an activator of adenylate cyclase. Using electrophoretic mobility shift assays and specific antibodies, we showed that CREB-1 binds specifically to the −79 to −52 region of the CD4 promoter. Last, we have identified two open reading frames (ORFs) of HHV-6, U86 and U89 from the immediate-early locus A, that can transactivate the CD4 promoter in HeLa cells. However, transactivation of the CD4 promoter by ORFs U86 and U89 is independent of the CRE element, suggesting that additional HHV-6 ORFs are likely to contribute to CD4 gene activation. Taken together, our results will help to understand the complex interactions occurring between HHV-6 and the CD4 promoter and provide additional information regarding the class of transcription factors involved in the control of CD4 gene expression.**

The CD4 antigen plays a role in both T-cell development and T-cell antigen recognition (24, 36, 37, 46). CD4, through interactions with the nonpolymorphic region of the class II antigen of the major histocompatibility complex (MHC), acts as an adhesion molecule that helps stabilize the complex formed by the T-cell receptor (TCR) and MHC class II antigen (5, 7, 13, 21). CD4 can also participate in T-cell activation through transmembrane signaling via the p56$^{lck}$ tyrosine kinase associated with its cytoplasmic tail (39, 43, 48, 49).

Control of CD4 gene expression is very complex. During the ontogeny of T cells, CD4 gene expression is turned on and off, depending on the stage of maturation of the T cell. A series of genetic elements, including the enhancer (4, 41, 45, 52), promoter (40), and silencer (6, 42, 44), are involved in control of the CD4 gene. The importance of these genetic elements for proper CD4 expression was demonstrated using transgenic animal models (4, 6, 42, 44). In fact, the presence of all regulatory sequences is required for proper tissue expression of the CD4 gene. Removal of any one of these elements influences either the level of expression or the cell type in which the gene is expressed. Maturation of double-positive CD4$^+$CD8$^+$ thymic cells into mature single-positive CD4$^+$CD8$^-$ or CD4$^-$CD8$^+$ peripheral T cells is a consequence of selective downregulation of the CD8 or the CD4 gene, respectively. Inhibition of CD4 gene expression in mature CD8$^+$ T cells occurs at the transcription level through the action of the CD4 silencer (42, 44). Very few experimental conditions were found to influence CD4 gene activation in mature CD8$^+$ T cells. Treatment of mature CD8$^+$ T cells with azacytidine (38), with the lectin concanavalin A (3), or with TCR agonists (9) was found to induce cell surface CD4 expression. Infection of mature CD8$^+$ T cells by human herpesvirus 6 (HHV-6) was also found to induce CD4 expression (26). The ability of HHV-6 to activate the CD4 gene was also demonstrated in natural killer (NK) cells (28) and in the hematopoietic progenitor cell line KG-1 (14). However, the molecular mechanisms leading to the expression of CD4 were never studied.

HHV-6 has been proposed to play a cofactorial role in progression to AIDS. Several observations prompted this hypothesis. (i) HHV-6 can infect CD4$^+$ T cells (2, 25, 27, 29, 47) and therefore contribute to the decline of such cell populations. (ii) HHV-6 can transactivate the long terminal repeat (LTR) of human immunodeficiency virus (HIV), thereby increasing HIV expression (8, 15, 19, 20, 30, 35, 50). (iii) HHV-6 is a potent inducer of tumor necrosis factor alpha secretion (10), an inflammatory cytokine known to activate HIV expression. (iv) HHV-6 can impair immunological functions such as T-cell proliferation (11, 18) and interleukin-2 synthesis (11). (v) HHV-6 can induce CD4 expression, thereby expanding the types of target cells susceptible to HIV infection (14, 26, 28). Although the latter is not likely to significantly contribute to disease, as HHV-6-infected cells will eventually die, the mechanisms implicated in CD4 gene activation by HHV-6 are of biological interest and will contribute to our understanding of the genetic control of the CD4 gene.

In the present work, we studied the interactions between HHV-6 and the CD4 promoter and have identified a functional transcription factor binding site belonging to the ATF/CRE family. This site is important in HHV-6-mediated trans-

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activation of the CD4 promoter. Furthermore, two genes of HHV-6 (U86 and U89) were found to transactivate the human CD4 promoter. The results presented provide new information regarding transcription factors interacting with the CD4 promoter and shed light on the mechanisms by which HHV-6 activates CD4 gene expression.

## MATERIALS AND METHODS

**Cells, culture conditions, and virus production.** HSB-2 cells were obtained from the NIH AIDS Research and Reference Reagent Program, and HeLa cells were obtained from the American Type Culture Collection (Manassas, Va.). HSB-2 cells were obtained as cultures in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and antibiotics. Cells were fed once every 4 to 5 days and seeded at a density of $3 \times 10^5$ cells/ml. HeLa cells were cultured in Dulbecco modified Eagle medium (D-MEM) supplemented with 10% FBS and antibiotics. Cells were passaged once a week. HHV-6 (GS strain) was propagated in HSB-2 cells and purified as described previously (10).

**Transfection and infection and luciferase determination.** HSB-2 cells were transfected by the DEAE-dextran method as previously described (12). After transfection, cells were centrifuged, washed, and infected with HHV-6 (multiplicity of infection, 0.1) for 2 h at 37°C. Cells were washed to remove unadsorbed virions and resuspended in complete medium. In experiments involving the drug phosphonoformic acid (PFA), cells were incubated for 1 h with 100 μg of PFA/ml prior to infection with HHV-6 and kept throughout the experiment. After 48 h, cells were harvested, washed, and lysed in 150 μl of cell culture lysis buffer (Promega, Madison, Wis.). Twenty microliters of extracts was added to 100 μl of luciferase assay reagents (Promega), and activity was determined by using a T20/20 luminometer (Turner Design, Calif.).

For cotransfection studies, HeLa cells were transfected by the lipofectamine reagent (Life Technologies, Grand Island, N.Y.). Cells were plated 1 day prior to transfection at $2 \times 10^5$/well in a 6-well plate. DNA (0.75 μg of reporter plasmids and 0.75 μg of effector plasmids) was mixed and incubated with 6 μl of lipofectamine reagent before being added to HeLa cells. After 5 h, complete D-MEM was added to the wells. Forty-eight hours posttransfection the medium was removed and cells were washed and lysed in cell culture lysis buffer. Luciferase activity in each sample was determined as described above. In some experiments, HeLa cells were transfected with reporter plasmids and were treated the next day with forskolin (10 μM) (Sigma) or the control diluent dimethyl sulfoxide (DMSO). After an additional 24 h, cells were harvested and luciferase activity was determined.

**Flow cytometry.** HSB-2 cells were infected with HHV-6 until signs of infection (ballooning) were observed (4 to 7 days) by photonic microscopy. Cells were harvested, washed, and resuspended in 100 μl of phosphate-buffered saline (PBS) containing 0.1% FBS. Phycoerythrin (PE)-labeled anti-human CD4 (Pharmingen) or PE-labeled isotype-matched control antibodies were added to the infected and uninfected cells for 1 h at 4°C. Cells were washed twice with 15 ml of cold PBS and fixed in PBS containing 1% paraformaldehyde. In parallel, HHV-6 infection was determined by using monoclonal antibody OHV-1 against gp106 of HHV-6 (Advanced Biotechnologies Inc., Columbia, Md.) and fluorescein-labeled goat anti-mouse immunoglobulin G antibodies. Percentages of cells expressing CD4 and HHV-6 were determined with a FACScalibur flow cytometer (Becton-Dickinson, Mountain View, Calif.) after the acquisition of 10,000 events.

**Cloning of the CD4 promoter and generation of reporter gene constructs.** The human CD4 promoter was cloned using a PCR approach. From the published sequence (40) (GenBank accession no. U01066) two primers were synthesized (sense, 5′-ATTACTGCAGCCTCAACTTCCTGGGCTC-3′, and antisense, 5′-TTCCTTCTGCAGAGTCGTGCT-3′) and used to amplify an 1,100-bp fragment of Jurkat cell genomic DNA using *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.). The blunt-ended PCR fragment was treated with T4 polynucleotide kinase and ligated into the *Sma*I site of the pGL3basic vector (Promega) to generate the −1076CD4p construct. The presence and orientation of the insert were determined by restriction endonuclease digestion and sequencing.

**Generation of 5′ deletion mutants of the CD4 promoter.** To generate 5′ deletion mutants of the CD4 promoter, the −1076CD4p plasmid was digested with *Nsi*I and *Kpn*I and electrophoresed through a 1% agarose gel. The 5.4-kb band was isolated using a QIAEX extraction kit (QIAGEN, San Diego, Calif.) and treated with S1 nuclease to remove the 3′ protruding ends. The plasmid was then treated with Klenow enzyme (Boehringer Mannheim, Indianapolis, Ind.), extracted with phenol, precipitated, and ligated using the fast ligation kit from Boehringer Mannheim. Ligated plasmids were used to transformed *Escherichia coli* DH5α cells, and recombinant clones were confirmed by restriction endonuclease digestion and sequencing. This construct was named −618CD4p. To generate −333CD4p, the −1076CD4p construct was digested with *Apa*I and *Kpn*I and the 5.1-kb band was isolated and purified as described above. The plasmid was treated with S1 nuclease, Klenow enzyme, and self ligated. Bacteria were transformed, and recombinants were identified as described above. The −71CD4p construct was generated by digesting −1076CD4p with *Nhe*I and *Pvu*II. The 4.9-kb band was isolated as described above and treated with Klenow enzyme to generate blunt ends. After ligation, bacteria were transformed and recombinants were selected with ampicillin (Boehringer Mannheim). The −71CD4p mutant clone was identified by digestion and sequencing. The −44CD4p clone was generated by digesting −1076CD4p with *Nhe*I and *Bln*I. The 4.9-kb band was isolated by gel electrophoresis, treated with Klenow enzyme, and self ligated. Mutant clones were identified by restriction endonuclease digestion and sequencing.

**Internal deletion mutant and site-directed mutagenesis of the CD4 promoter.** To generate the Δ-71−44CD4p mutant, the −1076CD4p construct was digested with *Pvu*II and *Bln*I. The 5.9-kb band was purified by gel electrophoresis, treated with Klenow, and self ligated. After bacterial transformation, clones were isolated and the internal deletion mutant was identified by restriction endonuclease digestion and sequencing.

Site-directed mutagenesis of the putative ATF/CRE site of the CD4 promoter was performed using the Chameleon mutagenesis kit, following the manufacturer's technical guidelines (Stratagene). Both the −1076CD4p and the −71CD4p constructs were mutated to generate −1076M5CD4p and −71M5CD4p. The mutation changed the ATF/CRE site (TGACGT) to a *Dra*I site (TTTAAA). Mutation was confirmed by digestion with *Dra*I and sequencing of the region of interest.

**Electrophoretic mobility shift assay.** Nuclear extracts from uninfected and HHV-6-infected HSB-2 cells ($10^8$) were prepared according to the method of Dignam et al. (5a). The sequences of the oligonucleotides used for CD4-CRE binding studies were: (sense) 5′-AGCTCCAGCTGGTGACGTTTGGGGCCG G-3′ and (antisense) 5′-CCGGCCCCAAACGTCACCAGCTGGAGCT-3′, which correspond to the −79 to −52 region of the CD4 promoter. The double-stranded oligonucleotides corresponding to the consensus CRE binding site (sense, 5′-AGAGATTGCCTGACGTCAGAGAGCTAG-3′) and the mutant CRE binding site (sense, 5′-AGAGATTGCCTGTGGTCAGAGAGCTAG-3′) were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif.). One hundred nanograms of the CD4-CRE probe was end labeled with [$^{32}$P]ATP using T4 polynucleotide kinase (New England Biolabs, Beverly, Mass.) and purified over a spin column (Boehringer Mannheim). Gel shift reactions were performed in a total volume of 20 μl as follows: 8 μg of nuclear extracts was preincubated for 5 min at room temperature with 2 μg of poly(dI-dC) (Boehringer Mannheim) in a binding buffer containing 20% glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM dithiothreitol, 250 mM NaCl, and 50 mM Tris-HCl (pH 7.5). Subsequently, 0.1 ng ($5 \times 10^4$ cpm) of the labeled probe was added to the mixture and incubated for an additional 30 min at room temperature. The complexes were resolved on a 4% nondenaturing acrylamide gel containing 2% glycerol and 0.5× TBE (45 mM Tris [pH 8.3], 45 mM boric acid, 1 mM EDTA) at room temperature for 2.5 h at 110 V. Competition experiments were performed by supplementing the reaction mixture with 20 ng (~200-fold molar excess) of unlabeled competitor probe. For the supershift experiments, nuclear extracts were preincubated for 1 h at 4°C in the presence of 2 μg of the indicated antibody (Santa Cruz Biotechnology), poly(dI-dC), and binding buffer. Subsequently, the labeled CD4-CRE probe was added, and the mixture was incubated at room temperature for 30 min before separation on gel.

**Western blot.** HSB-2 or HSB-2 cells infected with HHV-6 (multiplicity of infection, 0.1) for either 48 or 72 h were lysed in Laemmli SDS-PAGE sample buffer. Proteins ($2 \times 10^5$ cell equivalent) were separated by electrophoresis through an SDS–10% acrylamide gel and transferred onto polyvinylidene difluoride membranes. Blots were probe for either total CREB using rabbit anti-CREB antibodies (Santa Cruz Biotechnology) or for the phosphorylated form of CREB (P-CREB) using specific antibodies (Upstate Biotechnology, Lake Placid, N.Y.). Alkaline phosphatase-conjugated goat anti-rabbit antibodies were used as secondary antibodies for the detection of proteins made by chemiluminescence. Quantification of CREB and P-CREB protein levels was performed by laser densitometry analysis.

**Immediate-early genes of HHV-6.** Plasmids containing open reading frames (ORFs) 16/17 (pIEGP2-3′) and ORFs 18/19 (pIEG1-2) (35) from the IE-B region and a plasmid containing ORF 86 (pHV6U86) (34) from the IE-A region were generously provided by John Nicholas. pHV6U86 was generated by PCR using the following primer pair: sense, 5′-GAAGGACTCGTCTCCGG-3′ (genome coordinates, 125787 to 125771), and antisense, 5′-GGGTGCTATCACA TCAG-3′ (genome coordinates, 130936 to 130920). The 5.1-kbp fragment which contains the entire U86 ORF was cloned into the pSK+ pBluescript vector. The expression plasmid pBC-ORF, kindly provided by Michelle E. D. Martin, was generated by PCR using the following primer pair: sense, 5′-ACATCTAGGTT TCATCTAGC-3′ (genome coordinates, 133091 to 13072), and antisense, 5′-TT AAACATGTGACATATAAC-3′ (genome coordinates, 135713 to 135694). The 2.6-kbp PCR fragment, which contains the entire U89 ORF, was cloned into pBC/CMV as described previously (30).

## RESULTS

**Induction of cell surface CD4 expression in HHV-6-infected HSB-2 cells.** Previous reports have shown that infection of mature $CD8^+$ T cells (26), NK cells (28), and hematopoietic cells (14) by HHV-6 leads to CD4 gene expression. Confirmation of these results has now been extended to the immature,

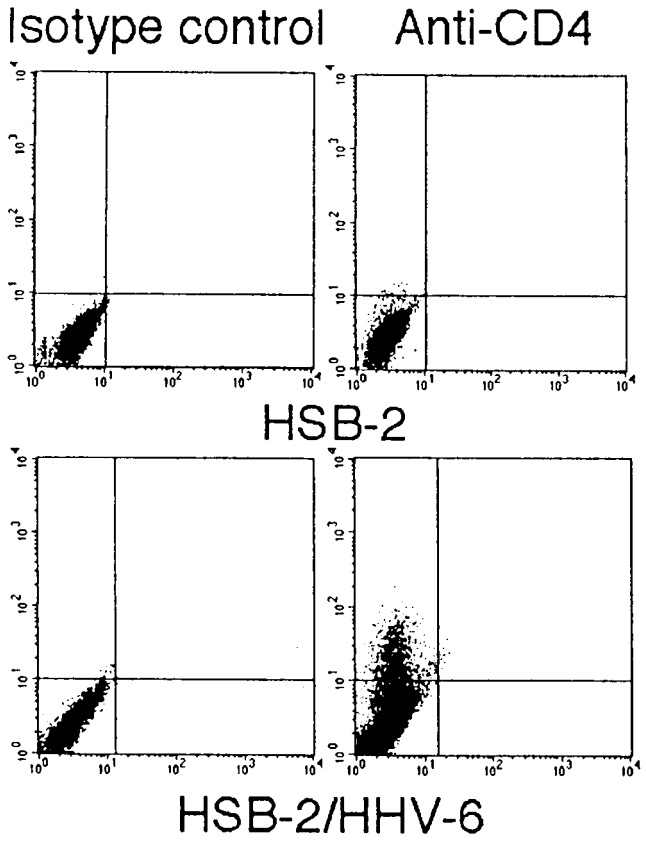


FIG. 1. Cell surface expression of CD4 after infection of HSB-2 cells with HHV-6. HSB-2 cells ($CD4^-$) were either treated with mock-infected culture fluid or infected with HHV-6 (GS strain). When signs of cytopathic effect were observed (5 to 7 days), cells were analyzed for CD4 surface expression by flow cytometry using PE-labeled anti-CD4 or isotype-matched control antibody. Top panels represent uninfected cells, while bottom panels depict HHV-6-infected cells.

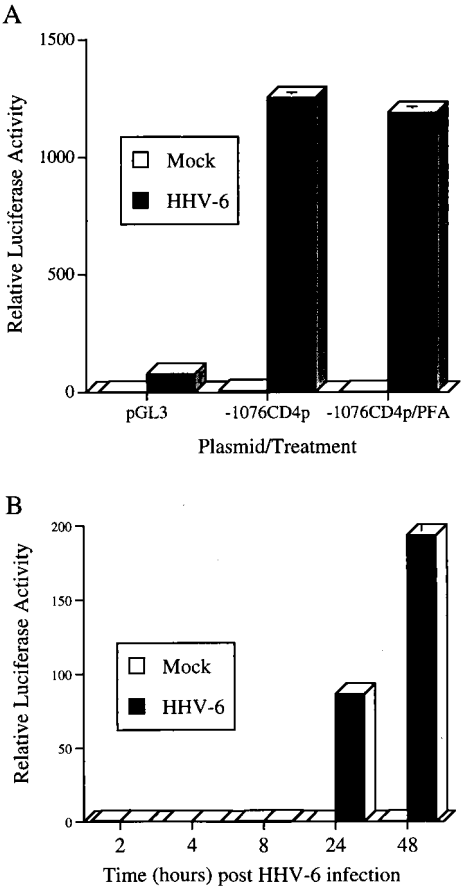


FIG. 2. (A) Activation of the CD4 promoter by HHV-6. HSB-2 cells were transfected with control (pGL3) or with −1076CD4p constructs and infected with HHV-6 or treated with mock-infected culture supernatant. PFA was added 1 h prior to infection, and PFA was kept throughout the experiment. Cells were harvested and lysed at 48 h postinfection, and luciferase activity was determined. Results (mean ± standard deviation), expressed as relative luciferase activity, are calculated from triplicate cultures and are representative of three experiments. (B) Kinetics of CD4 promoter activation by HHV-6. HSB-2 cells were transfected with the −1076CD4p construct and treated, the next day, with either mock-infected fluid or with HHV-6. At various times after infection, cell were harvested and lysed and luciferase activity was determined. Results (mean ± standard deviation) are calculated from triplicate cultures and are representative of three experiments.

$CD4^-$ T-cell line HSB-2. Ten percent of 7-day-old productively HHV-6-infected HSB-2 cells were found to express CD4 antigen at the cell surface (Fig. 1). This is in contrast to results for uninfected cells for which less than 0.1% of cells were found positive, by flow cytometry, for CD4 expression. Approximately 30% of HSB-2 cells were infected with HHV-6 as determined by immunofluorescence using antibodies specific for HHV-6 gp106 protein (not shown).

**Activation of the CD4 promoter by HHV-6.** CD4 gene regulation is regulated in a very complex manner. Several genetic elements, such as distal and proximal enhancers, a silencer, and a promoter, are known to play a role in the control of in vivo CD4 gene expression. Our present work focuses mainly on the interactions between HHV-6 and the CD4 promoter. To study the effects of HHV-6 on CD4 promoter activity, we cloned, upstream of a luciferase reporter gene, the 1,076-bp CD4 promoter in the promoterless pGL3basic vector to create the −1076CD4p plasmid.

HSB-2 cells were transfected in duplicate either with the pGL3basic vector or with the full-length CD4 promoter and subsequently infected with HHV-6. Two days after infection, luciferase activity was monitored. As shown in Fig. 2A, HHV-6 can minimally activate the pGL3basic vector. However, HHV-6 was able to strongly transactivate the full-length CD4 promoter with greater than a 10-fold increase in activity when compared to that of the pGL3basic vector.

It has been reported previously that HHV-6 can induce cell surface CD4 expression in the presence of the viral DNA polymerase inhibitor, (26). To test whether HHV-6 can activate the CD4 promoter in the presence of PFA, HSB-2 cells were transfected with the full-length CD4 promoter plasmid and infected with HHV-6 in the presence of PFA. Results (Fig. 2A) indicate that luciferase activity in the PFA-treated group was similar to that of the untreated, HHV-6-infected HSB-2 cells, suggesting that an immediate-early or early gene(s) of HHV-6 is sufficient for CD4 promoter transactivation. Effectiveness of PFA treatment was confirmed by the absence of HHV-6 glycoprotein B expression, a late viral gene product (data not shown).

**Kinetics of CD4 promoter activation by HHV-6.** In the next series of experiments, we performed kinetic analyses of CD4 promoter activation by HHV-6. HSB-2 cells were transfected with the full-length CD4 promoter and infected with HHV-6 on the next day. At various times after infection, cells were lysed and luciferase activity was determined. As shown in Fig.

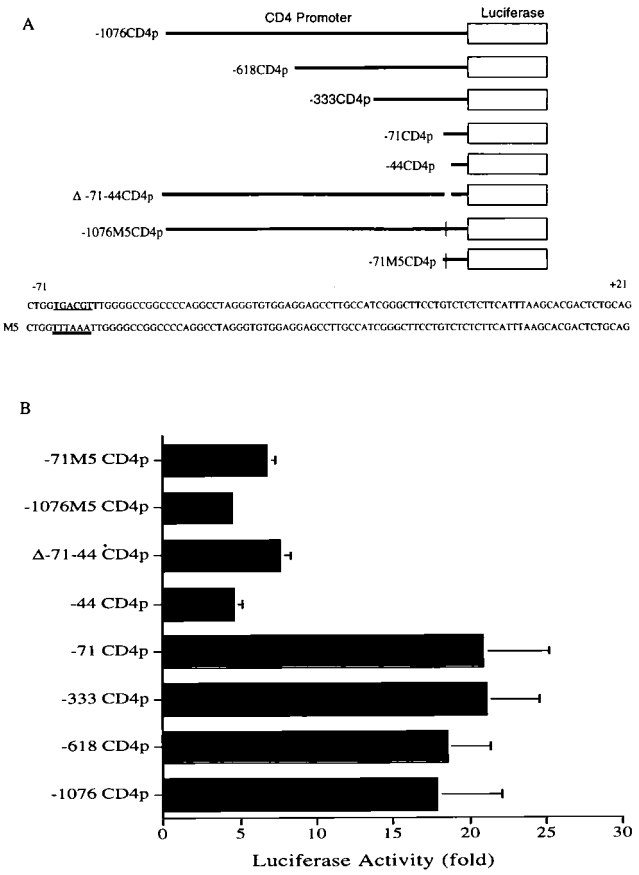


FIG. 3. (A) Schematic representation of wild-type (−1076CD4p) and mutant CD4 promoter luciferase reporter gene constructs. The cloning plasmid used for all of these constructs was the pGL3basic vector. The vertical line in constructs −1076M5CD4p and −71M5CD4p represents where the M5 mutation was introduced. The M5 mutation, which changes the putative ATF/CRE site (underlined) of the wild-type sequence to TTTAAATT (double underline), is shown at the bottom of the panel. (B) Identification of a minimal CD4 promoter responsive to HHV-6. HSB-2 cells were transfected with a series of mutants of the CD4 promoter and subsequently infected with HHV-6 or treated with mock-infected culture supernatant (mock). Forty-eight hours postinfection, cells were harvested and lysed and luciferase activity was determined. Results, from triplicate cultures, are representative of five experiments and are expressed as fold increase in luciferase activity after normalization with values from uninfected, transfected HSB-2 cells.

2B, there is no promoter activity during the first 8 h postinfection. However, at both 24 and 48 h postinfection, a strong increase in luciferase activity was detected. The fact that no activity was recorded during the initial phases of infection (2 to 8 h) suggests that CD4 promoter activation by HHV-6 is not simply the consequence of receptor-mediated transcriptional activation. In support of such hypothesis is the fact that UV-irradiated HHV-6, which allows binding of the virus to its receptor but prevents transcription of viral genes, failed to transactivate the CD4 promoter (data not shown).

**Identification of a minimal CD4 promoter responsive to HHV-6.** As represented in Fig. 3A, eight different CD4 promoter constructs were generated. These range from a full-length CD4 promoter (−1076CD4p) to a CD4 promoter containing only 44 nucleotides upstream of the transcription initiation site (−44CD4p). Also included is an internal deletion construct (Δ-71-44CD4p) and site-directed mutant promoters (−1076M5CD4p and −71M5CD4p).

We have shown in the first series of experiments (Fig. 2) that the CD4 promoter can be efficiently transactivated by HHV-6. To better identify which region(s) of the promoter is responsive to HHV-6, we tested a series of 5′ deletion mutants of the CD4 promoter. These constructs were independently transfected into HSB-2 cells, followed by infection with HHV-6. All constructs showed similar activity under basal conditions, i.e., in the absence of HHV-6. The −1076CD4p, −618CD4p, −333CD4p, and −71CD4p constructs were equally responsive to HHV-6 infection, as evidenced by the fold induction in luciferase activity (Fig. 3B). However, the removal of an additional 27 nucleotides from the −71CD4p plasmid, to create the −44CD4p plasmid, was found to significantly impair the ability of HHV-6 to activate the CD4 promoter. Indeed, a greater than fourfold decrease in luciferase activity is recorded with the −44CD4p plasmid compared to that of the −71CD4p vector.

**Identification of a ATF/CRE site important for transactivation by HHV-6.** The region located between −71 and −44 of the CD4 promoter is important for HHV-6-mediated transactivation (Fig. 3B). To determine whether other *cis*-acting elements play a role in HHV-6 transactivation of the CD4 promoter, we generated a construct in which the region from −71 to −44 was deleted, leaving the rest of the promoter intact (construct Δ-71−44CD4p, Fig. 3A). This construct was transfected in HSB-2 cells and tested for HHV-6 responsiveness. As shown in Fig. 3B, the Δ-71−44CD4p construct responded to HHV-6 with a reduced transactivation ability, as was the case with the −44CD4p construct. These results suggest that DNA sequences located elsewhere than within the −71 to −44 region do not play a role in the ability of HHV-6 to transactivate the CD4 promoter. Computer analysis of the −71 to −44 region reveals the presence of a putative ATF/CRE binding site. The region from −67 to −62 contains the sequence TGA CGT, which is identical to six of the eight nucleotides from a consensus ATF/CRE site (TGACGTCA). To determine whether this site is of importance for HHV-6 transactivation, we mutated the TGACGT site to TTTAAA (Fig. 3A). Mutation of this site was performed in both the wild-type CD4 promoter (−1076M5CD4p) and in the minimal −71CD4p (−71M5CD4p) construct. Mutated plasmids were transfected in parallel with the wild-type constructs and tested for responsiveness to HHV-6. The results (Fig. 3B) indicate that mutation of the putative ATF/CRE site of the CD4 promoter is detrimental for HHV-6 transactivation. In fact, the levels of activation are similar to those obtained with the constructs lacking the ATF/CRE site (−44CD4p and Δ-71−44CD4p) (Fig. 3B).

**Binding of transcription factors to the putative ATF/CRE sites of the CD4 promoter.** We demonstrated that the putative ATF/CRE site, located between −71 and −44 of the CD4 promoter is important for transactivation by HHV-6. Thus, we studied the binding of transcription factors to the −79 to −52 region of the CD4 promoter by electrophoretic mobility shift assay. Nuclear extracts from both uninfected and HHV-6-infected HSB-2 cells were obtained and used in binding assays. As shown in Fig. 4A, extracts from both sources efficiently bind the CD4 oligonucleotide, with no apparent differences in the intensity or pattern of binding. In addition, homologous competition with unlabeled CD4 oligonucleotide was very efficient. Equally efficient was a competition between the CD4 oligonucleotide and an oligonucleotide containing a wild-type CRE site. However, no competition could be observed with an oligonucleotide having a mutated CRE (M-CRE) consensus sequence.

In an effort to better characterize the protein complex binding to the CD4 oligonucleotide, we proceeded to perform

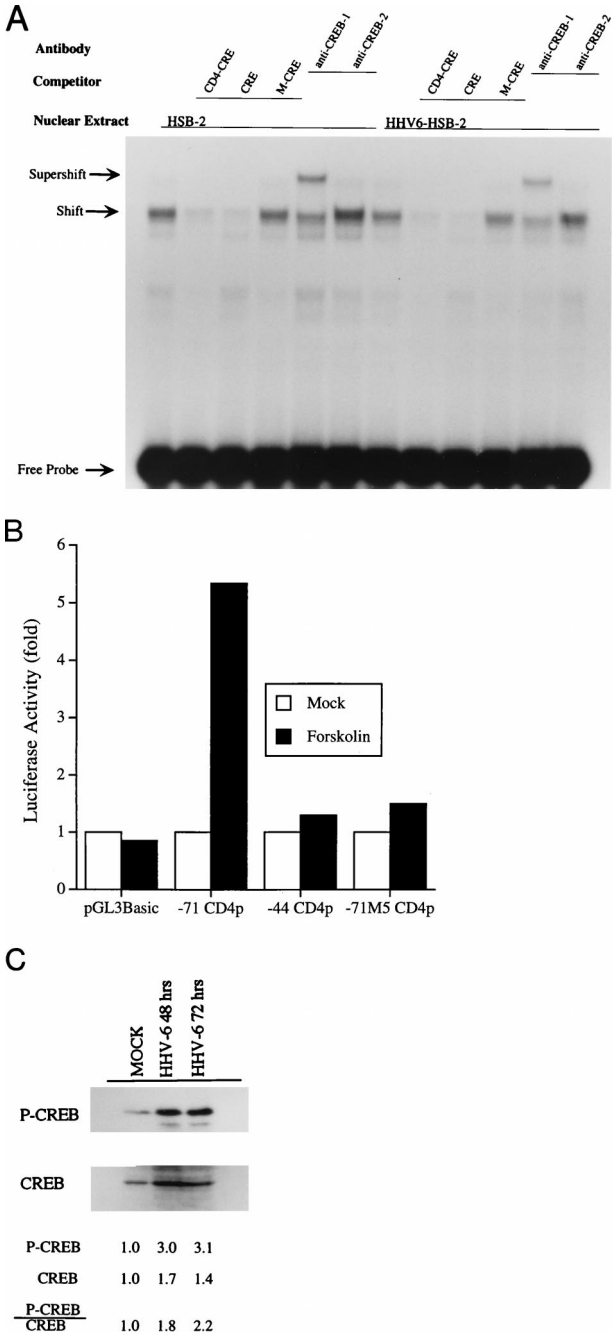


FIG. 4. (A) Binding of transcription factors to the ATF/CRE site of the CD4 promoter. Nuclear extracts from uninfected and HHV-6-infected HSB-2 cells were obtained and subjected to gel-shift assays, using the $^{32}$P-labeled probe spanning the −79 to −52 region of the CD4 promoter. Competition with a 200-fold excess of cold CD4 probe, wild-type ATF/CRE, and mutated ATF/CRE sequence was performed. Incubation of extracts with antibodies reacting with various transcription factors was also included. Results are representative of two experiments. (B) Activation of the CD4 promoter by forskolin. HeLa cells were transfected with either the pGL3basic, −71CD4p, −44CD4p, or −71M5CD4p construct and treated with DMSO (mock) or 10 μM forskolin. After 48 h, cells were harvested and luciferase activity was determined. Results, obtained from triplicate cultures, are expressed as fold induction of luciferase activity and are representative of three separate experiments. (C) Increased CREB phosphorylation in HHV-6-infected cells. HSB-2 and HHV-6-infected HSB-2 cells (48 and 72 h postinfection) were lysed in Laemmli buffer and analyzed for total CREB and P-CREB by Western blot using specific antibodies. Ratios of P-CREB/CREB were calculated for each sample following densitometric analysis of autoradiograms and normalization against CREB and P-CREB values of the mock-infected sample. Results are representative of two independent experiments.

supershift experiments using commercially available antibodies that react against known transcription factors. We first selected antibodies that were cross-reactive against several proteins known to bind DNA sequences having homologies to the putative ATF/CRE site found within the CD4 oligonucleotide. The antibodies used were capable of binding CREB-1/ATF-1/CREM-1, CREB-2/ATF-4, c-Jun/AP-1/JunB/JunD, and Ets-1/Ets-2 as negative controls. When used in the binding reactions, only the antibodies reacting with CREB-1/ATF-1/CREM-1 caused a supershift of the complex (data not shown). With the use of monospecific antibodies against CREB-1, we positively identified the CREB-1 protein as one of the factors binding to the ATF/CRE site of the CD4 promoter, as witnessed by reduced mobility (supershift) of the protein complex (Fig. 4A). Antibodies to CREB-2 were used as negative controls. Antibodies against CREM-1 were also unreactive (data not shown).

**Activation of the CD4 promoter by forskolin.** Transcriptional activation via ATF/CRE sites is often regulated through the action of protein kinase A (PKA) which phosphorylates and thereby activates transcription factors such as CREB (17, 22). Since PKA activity is influenced by levels of intracellular cAMP ($cAMP_i$), we tested whether an agonist such as forskolin, which causes an increase in $cAMP_i$, can activate the CD4 promoter. Transfected HeLa cells were stimulated with forskolin, and luciferase activity was determined. Results (Fig. 4B) indicate that the promoterless pGL3basic and −44CD4p constructs were not activated by DMSO (solvent) or by forskolin. However, the −71CD4p construct, which contains the ATF/CRE sequence, is efficiently activated by forskolin (fivefold). Treatment of cells with DMSO (mock) had no effect on promoter activity. The −71M5CD4p construct which contains a mutated ATF/CRE consensus site was not responsive to forskolin activation. These results suggest that the transcriptional activity of factors binding to the ATF/CRE site within the CD4 promoter can be regulated, at least in part, by cAMP and PKA.

**Analysis of CREB and phosphorylated CREB in HHV-6-infected cells.** Knowing that there is no difference in the binding pattern or in the amount of proteins bound to the −79 to −52 region of the CD4 promoter we studied, by Western blot, the levels of prosphorylated CREB (P-CREB) in extracts of HSB-2 and HHV-6-infected HSB-2 cells. Forty-eight and 72 h after infection the cells were harvested and lysed in SDS-PAGE buffer and proteins from $2 \times 10^5$ cell equivalents were separated by gel electrophoresis. After transfer onto a membrane, blots were probed with either anti-CREB or anti-P-CREB antibodies. Results indicate (Fig. 4C) that levels of P-CREB in the infected samples are twofold higher than those in the mock-infected cells when compared to the levels of unphosphorylated CREB. P-CREB levels of expression in HHV-6-infected cells were calculated after normalization against CREB and P-CREB levels of expression in the mock-infected samples.

**Identification of HHV-6 ORFs 86 and 89 as CD4 promoter transactivators.** From our kinetics data and the results obtained using the drug PFA, we deduced that virally encoded proteins belonging to the immediate-early or early class of protein were involved in CD4 promoter activation. In an effort to identify such proteins, we tested constructs containing ORFs from both the IE-A and IE-B regions of HHV-6. The plasmids contained U16/17 and U18/19 from the IE-B region and U86 and U89 from the IE-A region. In addition, a plasmid containing the transactivator U25 (35) was also tested. These plasmids were independently cotransfected into HeLa cells with the −71CD4p construct. HeLa cells were chosen for their much higher efficiency of transfection (15 to 20% as determined by flow cytometry following transfection with pGreen Lantern

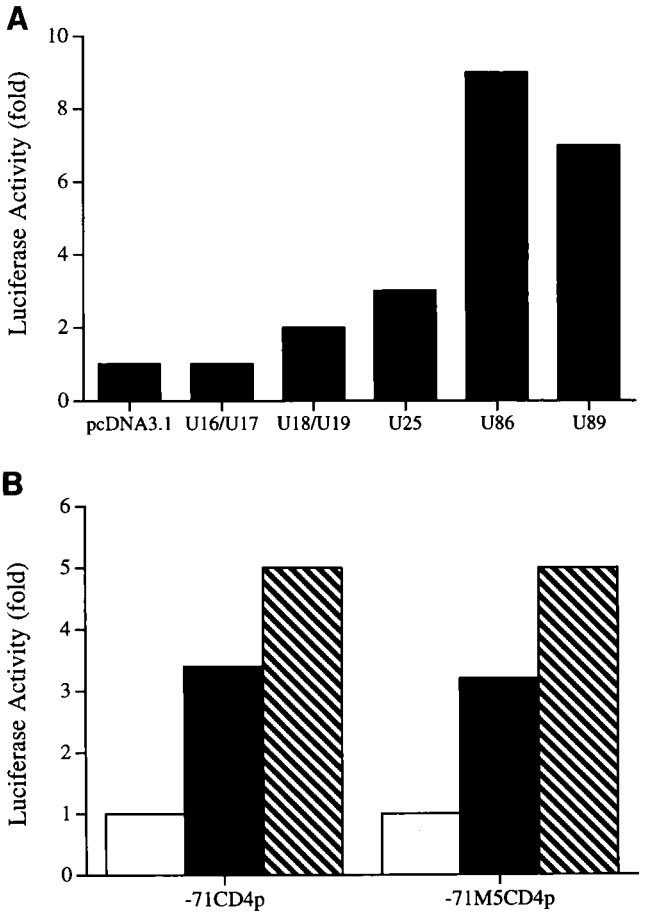


FIG. 5. (A) Transactivation of the CD4 promoter by genes of HHV-6. HeLa cells were cotransfected with either the control vector (pcDNA3.1) or with plasmids encoding HHV-6 genes (0.75 μg each) along with the −71CD4p (0.75 μg) construct. Forty-eight hours posttransfection, cells were lysed and luciferase activity was determined. Results are expressed as fold increase in luciferase activity and are representative of three independent experiments. (B) Effects of ATF/CRE mutation of transactivation by U86 and U89 of HHV-6. HeLa cells were cotransfected with either the pcDNA3.1 control vector or the U86 or the U89 construct along with the −71CD4p or the −71M5CD4p reporter plasmid. Forty-eight hours posttransfection, cells were lysed and luciferase activity was determined. The fold activation of pcDNA, U86, and U89 is represented by an open box, a hatched box, and a filled box, respectively. Results are representative of three independent experiments.

plasmid) than HSB-2 cells (<1%). The expression vector pcDNA3.1 was used as a negative control. Forty-eight hours posttransfection, cells were lysed and luciferase activity was determined. As shown in Fig. 5A, cells transfected with U16/U17, U18/U19, or the pcDNA3.1 control vector showed comparable low levels of luciferase activity. The plasmid containing U25 showed limited transactivation, with a 2.5-fold increase in reporter activity. Interestingly, the U86 and U89 constructs were capable of transactivating the −71CD4p promoter construct significantly, with a seven- to ninefold increase in activity. To determine whether the U86 and U89 transactivators were dependent on the presence of the ATF/CRE site to mediate their effects, we carried out similar experiments using the −71M5CD4p construct as the reporter plasmid. As shown in Fig. 5B, the U86 and U89 constructs were equally efficient in transactivating both the −71CD4p and the −71M5CD4p plasmids, suggesting an ATF/CRE-independent mode of action. The pcDNA3.1 vector was used as a negative control for the experiment and for the normalization of luciferase activity.

## DISCUSSION

T-cell precursors migrating out of the bone marrow and arriving in the thymus express low levels of CD4 ($CD4^{lo}CD8^-$) (51). These cells eventually lose CD4 expression and are referred to as double-negative ($CD4^-CD8^-$) thymocytes. During subsequent maturation steps, the CD4, CD8, and TCR genes are upregulated, forming a population of double-positive ($CD4^+CD8^+$) cells. Last, following positive and/or negative selection, survivor cells lose expression of CD4 or CD8, leading to a mature $CD4^+CD8^-$ or $CD4^-CD8^+$ T-cell phenotype as seen in the peripheral blood. Regulation of the CD4 gene is very complex and relies on the participation of several *cis*-acting elements, such as promoter, enhancer, and silencer. The original description of the human CD4 promoter indicates a lack of both TATA and CAAT boxes, no defined initiator sequence, and few transcription factor binding sites (40). Binding sites for and physical binding of Ets and Myb transcription factors to the CD4 promoter have been observed (40, 45). A minimal human CD4 promoter (nucleotides located at −40 to +16 relative to the transcription start site) with reduced activity compared to that of the full-length promoter was identified (40). These results suggested that additional *cis*-acting elements upstream of this minimal promoter are needed for full activity.

We began to study the human CD4 promoter during an attempt to identify the mechanisms by which HHV-6 induces CD4 gene expression in $CD4^-$ cells, such as mature $CD8^+$ T cells, NK cells, and a hematopoietic progenitor cell line. In general, once a $CD8^+$ T cell has reached maturity, it never expresses the CD4 antigen. In such cells, CD4 gene transcription is shut off through the action of the CD4 silencer located within the first intron of the CD4 gene (42, 44). The mode of action of the silencer is not completely understood. The silencer is not, however, the only element capable of exerting a negative regulatory action on the CD4 promoter. If such was the case, the CD4 promoter, in the absence of silencer sequences, would be active in many cell types. On the contrary, it has been reported that both the human and mouse CD4 promoters are highly active in $CD4^+$ cells and much less in $CD4^-$ cells (40, 45), suggesting that the CD4 promoter is tissue specific. One hypothesis is that promoter activity in various cell types may be influenced by the presence or absence of key transcription factors needed to fully activate the promoter.

When the effects of HHV-6 infection on CD4 promoter activity were tested, we noticed that this virus can very efficiently transactivate the CD4 promoter. CD4 promoter activation in HHV-6-infected HSB-2 cells (a $CD4^-$ immature T-cell line) was paralleled by cell surface induction of the CD4 antigen. The fact that, at any given time, we do not detect more than 10 to 15% of HSB-2 cells expressing surface CD4, although more than 30% are infected with HHV-6 as determined by immunofluorescence assay, suggests that CD4 induction may not be permanent. This is most likely the result of asynchronous lytic replication and cytopathic effects. Using 5′ deletion mutants of the CD4 promoter, we have identified a minimal promoter (from −71 to +21) responsive to HHV-6. This result suggests that *cis* elements upstream of this region are not involved in HHV-6 activation of the CD4 promoter. Removal of an additional 27 nucleotides greatly affected the ability of HHV-6 to activate the CD4 promoter. Analysis of the region from −71 to −44 revealed a partial ATF/CRE site (TGACGTTT) homologous to six of the eight nucleotides of

the wild-type consensus sequence (TGACGTCA). Mutation of the ATF/CRE site of the CD4 promoter to TTTAAATT or deletion of the −71 −44 region from the full-length promoter was also found to impair the ability of HHV-6 to transactivate the CD4 promoter.

ATF/CRE sites are present and are involved in the regulation of many cellular and viral promoters. Interestingly, the HHV-6 DNA polymerase promoter contains a single ATF/CRE site located at the −70 region that is critical for promoter activity (1). Removal or mutation of the ATF/CRE site eliminated the ability of HHV-6 to activate its own polymerase promoter (1). Furthermore, HHV-6 was shown to transactivate the Epstein-Barr virus Zebra promoter through a single ATF/CRE site (TGACATCA) located in the −67 to −60 region (12). The ATF/CRE site can bind numerous transcription factors belonging to the bZIP leucine zipper family of proteins, including ATF/CREB/CREM, Fos/Jun, and C/EBP (31). Our results, and those of Agulnik et al. (1) and Flamand and Menezes (12), indicate that constitutively expressed proteins bind to the ATF/CRE sites. To better characterize the interaction between HHV-6 and the CD4 promoter, we studied, using gel shift assays, the binding patterns of proteins from uninfected and HHV-6-infected HSB-2 cells. No difference in binding intensities or binding patterns between extracts from uninfected and HHV-6-infected HSB-2 cells could be detected, suggesting that viral transactivators do not bind DNA directly or that they are too limited in amount for detection. In order to identify protein(s) bound to the ATF/CRE site of the CD4 promoter within cell extracts, we made use of antibodies directed against several transcription factors known for their binding to ATF/CRE sites. Only one of these antibodies was found to be reactive for the complex bound to the CD4 promoter, identifying at least one of the factors binding to the ATF/CRE site as CREB-1. These results were further confirmed by using recombinant CREB-1 protein, which could efficiently bind to the ATF/CRE site within the CD4 promoter (data not shown).

Viral transactivators may interact with basal transcription units and adjacent transcription factors, bridging them together to allow efficient transcription to occur. In addition, some of the factors binding to the ATF/CRE site, such as CREB, efficiently bind DNA but are transcriptionally inactive until they become phosphorylated (17, 22). To help explain efficient CD4 promoter transactivation in the presence of constant levels of CREB-1 protein, we studied the levels of active CREB, i.e., phosphorylated CREB-1 in extracts from uninfected and HHV-6-infected HSB-2 cells. Using specific monoclonal antibodies, we were able to show increased levels of the phosphorylated form of CREB in HHV-6-infected cell extracts when compared to those of uninfected HSB-2 cells. Viral transactivators may therefore directly, or indirectly through activation of cellular kinases, promote transcription through the phosphorylation of key regulatory transcription factors.

Kinetics analyses suggest that direct transduction of signals resulting from the interactions of HHV-6 with its cellular receptor(s) is not responsible for CD4 promoter activation. More than 8 h of infection were needed for CD4 promoter activation, indicating, in all likelihood, that cellular and/or viral proteins outside the signaling cascade are needed. Supporting this hypothesis is the observation that UV-irradiated HHV-6, which can still bind to cells but cannot transcribe any of its genes, does not activate the CD4 promoter. To better characterize the gene(s) of HHV-6 involved in CD4 promoter activation, we made use of the drug PFA which restricts gene expression of the immediate-early and early classes of genes. The fact that PFA treatment of cells had no effect on the ability of HHV-6 to activate the CD4 promoter suggested that immediate-early or early genes were involved in promoter activation. The HHV-6 genome, which is essentially collinear with that of human cytomegalovirus (HMCV) (16, 23, 32), contains a long (141 kbp) unique coding region flanked by two directly repeated terminal sequences of approximately 8 kbp each (16). Within the unique segment, two loci encoding potential immediate-early genes have been identified. ORFs 16 to 19 of the immediate-early region B are homologous to UL36 to UL38 of HCMV (16, 35). Although these gene segments were found capable of transactivating the HIV LTR (35) none of these ORFs was able to transactivate the CD4 promoter. Region A, the second HHV-6 immediate-early locus contains U86 and U89, which are positional homologs of HCMV IE2 and IE1, respectively. In cotransfection experiments, U89 was shown capable of transactivating the HIV LTR (30). When we tested U86 and U89, both of these genes were found to be capable of activating the CD4 promoter. U86 has the capacity to code for a 152-kDa protein whose carboxy-terminal half has homology with IE2 (UL122) of HCMV (16). U89 does not share sequence homology with any HCMV proteins although a homologous gene is observed in the more closely related HHV-7 (33). U89 was capable of transactivating several promoters, including those having ATF sites such as the E4 early promoter of adenovirus (30). However, as is the case for the CD4 promoter, transactivation of the E4 promoter by U89 is independent of the ATF/CRE consensus sequence (30). Furthermore, transactivation of the HIV LTR by U89 of HHV-6 was found to be impaired if the NFκB, TATA box, or SP1 site was mutated (30). This suggests that U89 and perhaps U86 act as transcriptional enhancers through activation or recruitment of transcriptional unit complexes. The fact that multiple distinct transcription factor binding sites can be activated by U89 supports the hypothesis that this protein does not bind a specific DNA consensus sequence but rather interacts with regulatory proteins common to many transcriptional units.

HHV-6 has been associated with the transactivation of several viral promoters, including those Epstein-Barr virus (12) and HIV (8, 15, 19, 50). The present study indicates that HHV-6 can also efficiently transactivate the human CD4 promoter. A previously unrecognized ATF/CRE site within the CD4 promoter, capable of binding CREB-1, is important for promoter transactivation by HHV-6. Whether CD4 gene activation and *de novo* CD4 protein expression play a role in HHV-6 or HIV pathogenesis is unclear. CD4 gene activation could simply be a consequence of HHV-6 immediate-early and/or early gene expression. In fact, the promiscuity of CRE elements in viral and cellular promoters may lead to the activation of several genes sharing such common regulatory elements. The HHV-6 TATA-less DNA polymerase promoter is mainly controlled by a CRE element (1), suggesting that for efficient replication HHV-6 must have evolved mechanisms capable of activating transcription factors, such as CREB, involved in CRE-mediated promoter activation. Overall, our results provide a better understanding of HHV-6's biology and give new insights into the regulation of the human CD4 promoter.

## ACKNOWLEDGMENTS

Throughout these studies, Louis Flamand was supported by subsequent Fellowships from the Medical Research Council of Canada and from the National Health Research and Development Program of Canada. L.F. currently holds a Scholarship from the Fonds de la Recherche en Santé au Québec. This project was supported by a grant from the Medical Research Council of Canada to L.F. and by an RO3 (AI41854-02) grant from the NIH to M.S.R.

We thank Paolo Lusso for helpful discussions. We are grateful to John Nicholas and Michelle E. D. Martin for providing HHV-6 plasmids.

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