Activation of Monocyte Cyclooxygenase-2 Gene Expression by Human Herpesvirus 6

ROLE FOR CYCLIC AMP-RESPONSIVE ELEMENT-BINDING PROTEIN AND ACTIVATOR PROTEIN-1*

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Prostaglandin E₂ (PGE₂) is an arachidonic acid metabolite mainly produced by activated monocytes/macrophages (Mo/Mφ) that display broad immunomodulatory activities. Severeral viruses capable of infecting Mo/Mφ modulate PGE₂ synthesis in a way that favors the infection processes and the spread of virions. In the present work, we studied the effect of human herpesvirus 6 (HHV-6) infection of Mo/Mφ on PGE₂ synthesis. Our results indicate that HHV-6 induces COX-2 gene expression and PGE₂ synthesis within a few hours of infection. We mapped the different promoter elements associated with COX-2 gene activation by HHV-6 to two cis-acting elements: a cyclic AMP-responsive element and an activator protein-1 element. HHV-6 immediate-early protein 2 was identified as a modulator of COX-2 gene expression in Mo/Mφ. Finally, addition of PGE₂ to HHV-6-infected peripheral blood mononuclear cells cultures was found to increase significantly viral replication. Overall, these results further contribute to the immunomodulatory properties of HHV-6 and highlight a potential role for eicosanoids in the replication process of this virus.

Human herpesvirus 6 (HHV-6) was first isolated in 1986 from the peripheral blood of patients with AIDS or with lymphoproliferative disorders (1). HHV-6 was identified as the etiologic agent of exanthem subitum, a common childhood illness characterized by high fever and skin rash (2). A role for HHV-6 in diseases such as organ graft rejection (3), multiple sclerosis (4, 5), and AIDS (reviewed in Ref. 6) has also been suggested. One particular aspect of HHV-6 pathogenesis is its ability to infect cell types of hematopoietic origin. HHV-6 can infect T cells (7), B cells (1), NK cells (8), megakaryocytes (9), and monocytes/macrophages (10, 11). It is also suggested that HHV-6 can establish a latent infection in the monocyte/macrophage lineage (10). Following a primary infection, virus could only be recovered from macrophages suggesting that these phagocytes can serve as reservoir for lifelong persistence of HHV-6.

Monocytes/macrophages (Mo/Mφ) play a central role in immune response development by their ability to present antigens and secrete bioactive molecules. One such secreted product released by activated Mo/Mφ is prostaglandin E₂ (PGE₂) (12). PGE₂ is a potent lipid mediator of inflammation. The biosynthesis of PGE₂, an arachidonic acid metabolite, is tightly controlled by the activity of the cyclooxygenase (COX) enzymes (13, 14). There are two isoforms of the COX enzyme, COX-1 and COX-2, produced from differentially regulated genes. COX-1 is constitutively expressed in most tissues (15), whereas COX-2 is undetectable in normal tissues or resting immune cells, but its expression can be modulated by several stimuli (16). Human COX-2 gene promoter contains several sequences that have been shown to act as positive regulatory elements for the COX-2 gene transcription in different cell types (for a review see Ref. 17). The COX-2 promoter contains a classical TATA box, an E box, and binding sites for transcription factors such as nuclear factor-κB (NF-κB), nuclear factor-IL-6/CCAAT enhancer-binding protein, cyclic AMP-response element-binding protein (18), and nuclear factor-activated T cell/AP-1 (19).

Prostaglandins, particularly those of the E series, are widely regarded as pleiotropic immunomodulatory molecules, and the regulation of their expression appears to be critical for a number of immune responses. Several lines of evidence suggest that PGE₂, in addition to its proinflammatory function, may exert anti-inflammatory effects. For example, PGE₂ interferes with T lymphocyte responses by inhibiting the production of interleukin-2 (IL-2) (20), the major T cell growth factor. In this way, PGE₂ blunts the proliferation of T lymphocytes, a crucial step in the expansion of T cell clones. PGE₂ also inhibits the secretion of IFN-γ, a cytokine that has antiviral activity and is important in activating T cells and Mo/Mφ (21). Thus, PGE₂ inhibits the production of Th1-type cytokines (IFN-γ and IL-2),
switching the immune response toward a Th2-type cytokine profile (IL-4 and IL-5), having limited effectiveness in the development of an effective anti-viral response (22). Monocytes/macrophages, a major source of PGE2, are not refractory to its effects. PGE2 inhibits IL-1 synthesis and major histocompatibility complex class II expression in macrophages (23), limiting their ability to act as functional antigen-presenting cells (24, 25).

Many viruses that interact with Mo/Mϕ, including human immunodeficiency virus (HIV), human cytomegalovirus (HCMV), Epstein-Barr virus (EBV) and human T lymphotropic virus-1 (HTLV-1), efficiently modulate, positively or negatively, the synthesis of PGE2 (26–29). PGE2 synthesis modulation possibly represents a way that viruses have developed to alter the biological functions of these cells with an increase in viral replication and viral spread as the outcome. In fact, PGE2 is reported to enhance the replication of CMV, HIV-1, and HSV-1 (30–32).

The effects of HHV-6 infection on PGE2 synthesis and vice versa are unknown. Given the ability of HHV-6 to modulate immune functions (33–37), eicosanoid synthesis modulation could play an important role in the pathogenesis of this virus, especially during the early steps of an infection. In the present work, we dissected the mechanisms by which HHV-6 up-regulates PGE2 in monocytes/macrophages, and we identified the contribution of the different promoter elements in mediating COX-2 transcription. A candidate viral immediate-early gene was identified as a positive modulator of COX-2 gene expression. Finally, the effect of exogenously added PGE2 to HHV-6-infected PBMC cultures was determined.

MATERIALS AND METHODS

Cell Culture and Reagents—Peripheral blood mononuclear cells (PBMC) were obtained from healthy donors and isolated by centrifugation over lymphocyte separation medium. Monocytes were first enriched by centrifugation over a Percoll density gradient as described (38) and purified by cell-sorting procedure as described (28) (Epics Elite ESP; Coulter Electronics Canada, Burlington, Ontario, Canada). This procedure yielded >98% pure monocyte suspensions as determined by CD14 staining. The human cell lines Mono-Mac-1 and HeLa were cultured in RPMI and Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM glutamine, and antibiotics, respectively. All cell lines were tested and found to be free of mycoplasma contamination. Cyclosporin A was purchased from Novartis (Dorval, Quebec). PD 98059, KT-5720, U0124, U0126, SB 202190, and dicumarol were obtained from Calbiochem. The NS-398 was purchased from Cayman Chemical (Ann Arbor, MI).

FIG. 1. Effects of HHV-6 infection of human monocytes on PGE2 biosynthesis and COX protein expression. Enriched monocytes (98%) were incubated in the presence or absence of infectious HHV-6. A, cell-free supernatants were harvested at the indicated times and tested for the presence of PGE2 as described under “Materials and Methods.” Values (mean ± S.D.) are calculated from triplicate cultures and are representative of two experiments. B, COX-2 and COX-1 protein expression in HHV-6-infected monocytes was monitored over an 8-h period by Western blot, using specific anti-COX-2 and anti-COX-1 antibodies, as described under “Materials and Methods.” Equal amounts of proteins were loaded in all lanes as confirmed by actin blotting.

FIG. 2. Identification of cis-acting regions required for COX-2 promoter activation by HHV-6. Human monocytoid Mono-Mac-1 cells were transfected with the indicated COX-2 promoter constructs and cultured in the absence or presence of infectious HHV-6 for 24 h and assayed for luciferase (LUC) activity. The means of triplicate determinations, expressed as relative luciferase units (RLU ± S.D.), are shown. Results of a representative experiment out of three performed are shown. Cis-acting consensus sequences are denoted by boxes, and promoter regions, relative to the transcription initiation start site, are indicated in parentheses.
Viral Preparation—The HHV-6 (type B, Z29 strain) used in this study was propagated on Molt-3 cells as described (39). After 7 days of infection, virus was concentrated from culture supernatant by centrifugation (38,800 x g, 2 h 40 min) and resuspended in a minimal volume of complete culture medium. HHV-6 preparation had a titer of 6 x 10^6 infectious particles/ml.

Enzyme Immunoassays for PGE2—Supernatants from mock-treated and HHV-6-infected monocytes cultures were harvested at the indicated times and tested for the presence of PGE2 using a commercially available enzyme immunometric assay (Cayman Chemical, Ann Arbor, MI). The detection limit for PGE2 was 29 pg/ml, with less than 0.01% cross-reactivity for other PGs.

Western Blot Analysis—At the indicated time, mock-treated and HHV-6-infected monocytes were washed with phosphate-buffered saline and lysed in Laemmli buffer, boiled, and electrophoresed on a 10% SDS-polyacrylamide gel, and separated proteins were transferred onto polyvinylidene difluoride membranes. Membranes were incubated in 5% (w/v) dry milk in TBS-T saline (0.25 M Tris-HCl, pH 7.4, 5 mM KCl, 0.1 M NaCl, 0.1% Tween 20) for 30 min to block nonspecific sites. Blots were then incubated for 1 h in blocking solution containing either anti-COX-2 (1/1000), anti-COX-1 (1/500), or anti-actin (1/500) antibodies (Cayman Chemical, Ann Arbor, MI). Membranes were washed twice with TBS-T and treated with either a horseradish peroxidase-linked goat anti-mouse or anti-rabbit antibody (1/10,000). Reactive proteins were visualized by enhanced chemiluminescence (ECL) (PerkinElmer Life Sciences).

Plasmid Constructs—Human COX-2 promoter constructs were kindly provided by Dr. M. Fresno (Madrid, Spain) and have been described previously (19). The NFAT-inhibiting pVIVIT-GFP expression vector was supplied by Dr. Anjana Rao (Harvard Medical School, Boston) (40). The VIVIT-GFP fragment was excised from this vector and cloned in the pRc/Actin expression vector with HindIII/NotI restriction sites (pRc/Actin VIVIT-GFP) (41). KCREB, an expression vector coding for a dominant-negative form of CREB, was obtained from Dr. R. H. Goodman (42). The pcDNA3-flagMEKK1(K432M) vector was supplied by Dr. Tom Maniatis (Harvard University, Cambridge) (43). pBK-IE2A expression vector was generated by cloning of a full-length cDNA for IE2.2

Transfection and Luciferase Assays—Transfection of the human Mono-Mac-1 and HeLa cells was performed using DEAE-dextran and calcium phosphate transfection procedures, respectively. Mono-Mac-1 cells were washed once in a TS buffer (25 mM Tris-HCl, pH 7.4, 5 mM KCl, 0.6 mM NaHPO4, 0.5 mM MgCl2, and 0.7 mM CaCl2) and resuspended in 1 ml of TS buffer containing 10 μg of the indicated plasmids and 500 μg/ml DEAE-dextran. The mixture was incubated for 5 min at room temperature and 20 min at 37 °C. Thereafter, cells were diluted with 5 ml of complete culture medium supplemented with 100 μM chloroquine. After 45 min of incubation at 37 °C, cells were centrifuged.
resuspended at 1 x 10^6 cells/ml in complete culture medium, and incubated at 37 °C. After overnight incubation, cells were mock-treated or infected with HHV-6 for 4 h, then washed with phosphate-buffered saline, and resuspended in complete medium for an additional 24 h. HeLa cells were plated (75,000/well) the day before transfection in 24-well plates. Five μg of total DNA was added per well. pcDNA vector (Invitrogen) was used to normalize DNA amounts. After overnight incubation, transfection medium was replaced with complete medium. Cells were incubated for an additional 24 h at 37 °C. Luciferase activity was determined following lysis of cells in 1 x luciferase assay buffer (Promega, Madison, WI) using a MLX Microtiter Plate Luminometer (Dynex Technologies Inc., Chantilly, VA).

**mRNA Analysis**—Total RNA was isolated from mock- or HHV-6-infected monocytes using the TRIzol reagent (Invitrogen). Total RNA was treated with 2 units of RNase-free DNase for 30 min, phenol-extracted, and ethanol-precipitated. RNA (1 μg) was reverse-transcribed into cDNA using random hexamers and used for PCR amplification using primers specific for IE2 exon 2 (5'-CGA TCC AGT GGT GGA AGA AT-3') and exon 3 (5'-CGT CCG CAT GAT GTA GTC-G-3'). The PCR was amplified by 35 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min. Amplified cDNAs were separated by agarose gel electrophoresis. Amplions were transferred by capillary diffusion onto a nylon membrane. DNA was cross-linked to membrane by UV exposure (1200 J). Membrane was incubated at 42 °C before being exposed to film.

**DNA Dot Blot**—PBMCs were stimulated with PHA (1 μg/ml) for 3 days. Cells were mock- or HHV-6-infected for 2 h. Cells were resuspended in 400 μl of ice-cold buffer A (10 mM Hepes, pH 7.6, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) supplemented with protease inhibitors. After a 15-min incubation on ice, cells were lysed by the addition of 0.6% (v/v) Nonidet P-40. The nuclei were isolated by centrifugation, and proteins were extracted with 50 μl of buffer C (20 mM Hepes, pH 7.6, 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) containing protease inhibitors. Nuclear extracts were collected and stored at -80 °C. Protein concentration was determined by the BCA assay (Pierce). Nuclear extracts (3-5 μg) were incubated with poly(dI-dC) (0.2 mg/ml) and bovine serum albumin (1 mg/ml) in a DNA binding buffer (10 mM Hepes, pH 7.6, 4% glycerol, 1% Ficoll, 25 mM KCl, 1 mM dithiothreitol, 0.5 mM EDTA, 25 mM NaCl) on ice 10 min. Then, 60,000 cpm of 32P-labeled double-stranded oligonucleotides (CRE or AP-1) were added to the mixture and incubated at room temperature for 20 min. In the competition experiment, a 100-fold molar excess of unlabeled oligonucleotides was added to the binding reaction mixture 10 min prior to probe addition. In supershift experiment, 1 μg of antibodies against CREB-1 protein, c-JUN (Santa Cruz Biotechnology), or purified rabbit IgG (negative control) were mixed with samples for 10 min prior to probe addition. DNA protein complexes were resolved by nondenaturing PAGE.

**Results**

**HHV-6 Induces COX-2 Protein Expression and PGE\textsubscript{2} Biosynthesis in Human Monocytes**—Several studies (26–29) have described how viruses that are able to infect human monocytes do modulate PGE\textsubscript{2} synthesis. To establish whether HHV-6 affected this lipid mediator pathway, we monitored biosynthesis of PGE\textsubscript{2} in uninfected and HHV-6-treated monocyte cultures over an 8-h period. When monocytes were infected with HHV-6, PGE\textsubscript{2} levels increased gradually (Fig. 1A) with detectable synthesis by 2 h and maximal activity recorded 8 h post-infection. Two enzymes, COX-1 and COX-2, can be responsible for PGE\textsubscript{2} synthesis. To discriminate which isoforms of COX are most likely involved in PGE\textsubscript{2} synthesis following HHV-6 infection, we performed
Western blots for these proteins. First, for individual samples to be compared, we needed to assess the relative amounts of proteins loaded for each. The actin content was estimated and found to be equal for each sample (Fig. 1B). Second, the samples were analyzed for COX-2 and COX-1 protein expression. As shown in Fig. 1B, mock-treated monocytes do not constitutively express the COX-2 protein. However, COX-2 levels became detectable at 2 h post-infection and continued to increase up to 8 h, correlating directly with the release of PGE2. Levels of COX-1 isoform were not affected by HHV-6 (Fig. 1B). To confirm that COX-2 is the COX isoform responsible for HHV-6-related PGE2 production, we performed the experiment in the presence of NS-398, a selective COX-2 inhibitor (45). A dose of 1 μM NS-398 resulted in a 97% reduction in HHV-6-related PGE2 production (data not shown) indicating that production of PGE2 in HHV-6-treated monocytes is directly linked to the induction of the COX-2 protein following infection.

Analysis of COX-2 Promoter Activity in HHV-6-infected Monocytes—Depending of the cell type, several promoter elements were reported to play an important role in regulating COX-2 gene transcription (19, 46–60). Given the fact that monocytes are difficult to transfect in a reproducible fashion, we first analyzed COX-2 promoter activation in HHV-6 infected Mono-Mac-1. Mono-Mac-1 is a human cell line with properties of blood monocytes, which can be used as a model system to study monocytic functions in vitro (61). Compared with the promoterless vector (i.e. PXP-2), the COX-2 promoter (P2-1900) was significantly activated during HHV-6 infection (200-fold) compared with non-infected cells (Fig. 2). To map the regions responsible for this induction, we performed transient transfection experiments using deletion mutants of the COX-2 promoter constructs (Fig. 2). Transfection experiments with dele-
tion constructs indicate that promoter elements between −88 and +104 (P2-192) were necessary and sufficient to induce strong luciferase activity after HHV-6 infection. The P2-150 construct containing only a TATA box as the promoter element was minimally activated by HHV-6. These results suggest that the proximal NFAT (pNFAT)/AP-1 and/or CREs within the −88/+104 region is/are likely responsible for promoter activation following HHV-6 infection.

Identification of Cis-acting Regions Required for COX-2 Promoter Activation—Three regulatory elements, including pNFAT, AP-1, and CRE, are located within the −88/+104-bp region of the COX-2 promoter. Because of the importance of this region for the inducibility of the promoter by HHV-6, we determined the contribution of these sites to the overall transcriptional regulation of the promoter by using constructs (P2-274 and P2-192) containing specific mutations within the dNFAT, pNFAT, AP-1, or CRE sites. Details on the wild type and specific mutants are presented in Fig. 3A. The pNFATmut construct has both NFAT and AP-1 sites mutated, whereas in the CREmut construct, only the CRE site was modified. Transient transfection experiments with these plasmids indicated that mutation of the dNFAT element resulted in a strong

![Image of gel shift assays](https://example.com/gel-shift-assays.png)

**Fig. 6. Increased AP-1 and CRE binding proteins in nuclear extracts of HHV-6-infected monocytes.** Nuclear extracts from purified monocytes infected or not with HHV-6 were analyzed by electrophoretic mobility shift assay. The specific HHV-6-induced complexes are indicated by arrows. Gel shift assays were performed using oligonucleotides corresponding to the following: A, AP-1 consensus sequence, and B, CRE consensus sequence. A 100-fold molar excess of unlabeled AP-1, CRE, or NFκB consensus oligonucleotides was added to the binding reaction mixtures to determine binding specificity. C, nuclear extracts from purified monocytes mock- or HHV-6-infected were incubated with anti-CREB-1, anti-c-JUN, or with rabbit IgG prior to CRE and AP-1 32P-labeled probe addition. Results are representative of two independent experiments.
induction of the promoter, as expected (Fig. 2). Mutation of the pNFAT/AP-1 or the CRE element severely diminished the HHV-6-induced promoter activity (Fig. 3B). Our results therefore suggest that both CRE and pNFAT/AP-1 elements are involved for COX-2 promoter activation by HHV-6.

Effects of Dominant Repressor of NFAT, AP-1, and cAMP-responsive Element-binding Protein on HHV-6 Activation of COX-2 Promoter—To discriminate which of the CRE, NFAT, and AP-1 elements are essential for COX-2 promoter activation by HHV-6, we first cotransfected Mono-Mac-1 cells with P2-1900 promoter construct along with increasing amounts of an expression plasmid coding for a dominant-negative of NFAT (pRc/actin-VIVIT-GFP) followed by infection with HHV-6. As shown in Fig. 4A, HHV-6 is able to activate the COX-2 promoter in the presence of increasing amounts of a dominant-negative of NFAT, suggesting that this transcription factor is not implicated in the HHV-6-related COX-2 promoter activation. The quantity of VIVIT used corresponds to those that can totally abrogate IL-2 promoter activation in Jurkat T cells (data not shown). The transactivation of genes through the CRE was proposed to occur by the binding and phosphorylation of the transcription factor CREB (CRE-binding protein) (62, 63). To determine the contribution of CREB in the HHV-6-dependent COX-2 promoter activation, we used a double negative mutant designated KCREB (killer CREB). This dominant repressor of the wild type factor is unable to bind to CRE DNA sequence and blocks the ability of CREB to bind to the CRE when present as a KCREB:CREB heterodimer (42). Mono-Mac-1 cells were thus cotransfected with the P2-1900 promoter construct along with increasing amounts of the expression plasmid KCREB followed by infection with HHV-6. As shown in Fig. 4B, a dose-response inhibition of COX-2 promoter activity was recorded with increasing amounts of KCREB vector. This result suggests that CREB proteins are involved in the activation of COX-2 promoter by HHV-6. MEKK-1 is a mitogen-activated protein kinase that is involved in AP-1 activation through the intermediary of the SAPK/JNK kinase. To determine whether AP-1 regulates COX-2 promoter activity, we cotransfected Mono-Mac-1 cells with P2-1900 promoter construct along with increasing amounts of an expression plasmid coding for a dominant-negative of MEKK-1 (pDNA3-flag MEKK-1(K432M)) followed by infection with HHV-6. MEKK-1(K432M) inhibited the HHV-6-related COX-2 promoter induction in a dose-dependent manner (Fig. 4C) suggesting that AP-1 also plays a role in the activation of the COX-2 promoter by HHV-6.

Effects of Protein Kinase Inhibitors on the HHV-6-related COX-2 Promoter Activation—So far, our results suggest that CREB and AP-1 proteins play a major role in COX-2 activation by HHV-6 without the involvement of NFAT. To confirm that NFAT is not involved, Mono-Mac-1 cells were transfected with the P2-1900 reporter and incubated with cyclosporin A (CsA), a well-characterized calcineurin inhibitor (64–66), prior to HHV-6 infection. Our results (Fig. 5A) indicate that COX-2 promoter activation is as efficient in the absence or in the presence of infectious HHV-6 for 8 h. Analysis of IE2 mRNA expression was evaluated by reverse transcriptase-PCR and Southern blotting of PCR product as described under “Materials and Methods.” Results are representative of two independent experiments.
in COX-2 induction by HHV-6. CREB transcriptional activity is regulated by its phosphorylation on Ser133 by protein kinase A enzymes. To confirm our results obtained using the KCREB dominant-negative expression vector, Mono-Mac-1 cells were transfected with the P2-1900 reporter and treated with KT-5720, a potent cAMP-dependent protein kinase inhibitor (67, 68) prior to and during infection with HHV-6. Our results clearly show a dose-dependent inhibition of promoter activity with KT-5720 (Fig. 5B), corroborating the involvement of CREB in the process. Our results using the dominant-negative form of MEKK1 also suggest that the c-Jun N-terminal kinase (SAPK/JNK) pathway is involved in COX-2 gene activation. Mitogen-activated protein kinase (MAPK) pathways mediate the regulation of COX-2 expression to a variety of stimuli (69–71). Three related MAPK cascades have been described (72, 73) and include the ERK pathway, the SAPK/JNK pathway, and the p38MAPK pathway. To determine which kinase activation was required for HHV-6-induced COX-2 transcription, Mono-Mac-1 cells were transfected with P2-1900 and subsequently treated with MEK/ERK (U0124 (74) and PD 98059 (75–78)), p38MAPK (SB 202190 (79)), and SAPK/JNK (dicumarol) (80, 81) inhibitors prior to and during infection with HHV-6. Our results show that treatment with MEK/ERK (Fig. 5C) and p38 inhibitors (Fig. 5D) did not affect the induction of COX-2 promoter by HHV-6, whereas dicumarol was able to reduce, in a dose-dependent manner, COX-2 induction by HHV-6 (Fig. 5E). Dicumarol was also able to significantly prevent COX-2 protein induction in HHV-6-infected monocytes (data not shown). These results confirm that MEKK1/SAPK/JNK pathway is engaged during HHV-6 infection, possibly leading to AP-1 activation and COX-2 promoter transcription.

**HHV-6 Activates AP-1 and CREB Transcription Factors Regulating COX-2 Expression**—To confirm further the role of AP-1 and CRE elements in mediating HHV-6-related COX-2 transcription, electrophoretic mobility shift assays were performed using end-labeled oligonucleotide probes containing the AP-1 or CRE consensus binding sequences. Results indicate an increase in both AP-1 (Fig. 6A) and CRE (Fig. 6B) binding activity in nuclear protein extracts of HHV-6-infected monocytes compared with the mock-treated cells. The DNA complexes were efficiently competed with a 100-fold molar excess of unlabeled CRE or AP-1 but not significantly by heterologous NFκB consensus oligonucleotides (Fig. 6, A and B). To identify the proteins bound to CRE and AP-1, we performed supershift assays. Nuclear extracts were incubated with anti-CREB-1, anti-c-JUN, or irrelevant antibodies (IgG) prior to labeled probe addition. With the AP-1 probe, only the c-JUN antibodies caused a supershift of the complex (Fig. 6C, left panel). By using the CRE-labeled probe, only the anti-CREB-1 antibodies supershifted the complex (Fig. 6C, right panel). These results further support a role for c-JUN and CREB-1 in COX-2 gene activation following infection of monocytes by HHV-6.

**Effect of Inactivated Virus on COX-2 Promoter Activation**—The results presented so far have focused on cellular kinases and transcription factors involved in COX-2 gene activation. In an attempt to characterize the viral factor associated with the COX-2 promoter induction, we first transfected cells with P2-1900 promoter construct and then incubated these cells in the presence of UV- or heat-inactivated HHV-6. UV irradiation causes DNA damage and prevents viral gene transcription with minimal effect on viral particle structural integrity, whereas heat causes proteins denaturation and affects viral particle integrity. As shown in Fig. 7, UV-treated and heat-inactivated HHV-6 did not activate the COX-2 promoter, suggesting that binding to the cell surface and entry of virus into the cells are not sufficient to activate the COX-2 gene. These results strongly suggest that viral gene transcription is required to observe COX-2 promoter activation.

**IE2 Protein of HHV-6 Is Implicated in COX-2 Promoter Activation**—A characteristic of betaherpesvirus IE gene products includes their ability to transactivate homologous and heterologous promoters (82–87). A number of HHV-6 gene products have been described as potential transcriptional transactivators, and some of these have been classified into two distinct regions, IE-A (IE1 and IE2) and IE-B (U16–U19) (88–90). By having determined that COX-2 protein can be induced as rapidly as a 2-h post-HHV-6 infection of monocytes/macrophages, we focused our efforts on IE genes. A previous report (84) has suggested that HHV-6 U86 and U89 were capable of transactivating the human CD4 promoter through a CRE element. We therefore tested the effects of IE1 and IE2 expression vector on COX-2 promoter activity. Our results show that IE2 is very efficient in transactivating COX-2 promoter (Fig. 8A). Compared with IE2, IE1 was a much weaker activator of CRE-containing reporter vector (data not shown). Finally, we performed HHV-6 IE2 mRNA detection in infected monocytes/macrophages. A strong and specific signal of IE2 could be detected in 8-h infected monocytes/macrophages (Fig. 8D). During this time, IE1 could also be detected (data not shown). These results suggest that HHV-6 IE2 protein is likely to play a role in COX-2 gene activation during the early step of infection.

**Effects of Exogenous PGE2 on HHV-6 Infection**—PGE2 was reported to positively modulate infections by several viruses such as CMV, HIV, and HSV-1 (30–32). In order to assess whether PGE2 could influence the replication of HHV-6, we incubated freshly infected PBMC cells with 1 μM PGE2 and analyzed by dot blot assay the levels of HHV-6 viral DNA. As shown in Fig. 9, HHV-6 DNA levels were increased in PGE2-treated cells compared with that of untreated cultures. PhosphoImager quantitation indicates a more than 2.5-fold enhancement in viral DNA level in PGE2-treated cultures. To

![Fig. 9. PGE2 induces HHV-6 replication in PBMC. PHA-activated PBMC were mock-infected or infected with HHV-6 in the absence or presence of PGE2 (1 μM). 4 days post-infection, total DNA was isolated as described under “Materials and Methods” and analyzed by dot blot by using a 32P-labeled HHV-6 DNA probe. Results are representative of two independent experiments.](http://www.jbc.org/)

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**HHV-6 Induction of PGE2 Synthesis**

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**Effect of Inactivated Virus on COX-2 Promoter Activation**—The results presented so far have focused on cellular kinases and transcription factors involved in COX-2 gene activation. In an attempt to characterize the viral factor associated with the COX-2 promoter induction, we first transfected cells with P2-1900 promoter construct and then incubated these cells in the presence of UV- or heat-inactivated HHV-6. UV irradiation causes DNA damage and prevents viral gene transcription with minimal effect on viral particle structural integrity, whereas heat causes proteins denaturation and affects viral particle integrity. As shown in Fig. 7, UV-treated and heat-inactivated HHV-6 did not activate the COX-2 promoter, suggesting that binding to the cell surface and entry of virus into the cells are not sufficient to activate the COX-2 gene. These results strongly suggest that viral gene transcription is required to observe COX-2 promoter activation.

**IE2 Protein of HHV-6 Is Implicated in COX-2 Promoter Activation**—A characteristic of betaherpesvirus IE gene products includes their ability to transactivate homologous and heterologous promoters (82–87). A number of HHV-6 gene products have been described as potential transcriptional transactivators, and some of these have been classified into two distinct regions, IE-A (IE1 and IE2) and IE-B (U16–U19) (88–90). By having determined that COX-2 protein can be induced as rapidly as a 2-h post-HHV-6 infection of monocytes/macrophages, we focused our efforts on IE genes. A previous report (84) has suggested that HHV-6 U86 and U89 were capable of transactivating the human CD4 promoter through a CRE element. We therefore tested the effects of IE1 and IE2 expression vector on COX-2 promoter activity. Our results show that IE2 is very efficient in transactivating COX-2 promoter (Fig. 8A). Compared with IE2, IE1 was a much weaker activator of CRE-containing reporter vector (data not shown). Finally, we performed HHV-6 IE2 mRNA detection in infected monocytes/macrophages. A strong and specific signal of IE2 could be detected in 8-h infected monocytes/macrophages (Fig. 8D). During this time, IE1 could also be detected (data not shown). These results suggest that HHV-6 IE2 protein is likely to play a role in COX-2 gene activation during the early step of infection.

**Effects of Exogenous PGE2 on HHV-6 Infection**—PGE2 was reported to positively modulate infections by several viruses such as CMV, HIV, and HSV-1 (30–32). In order to assess whether PGE2 could influence the replication of HHV-6, we incubated freshly infected PBMC cells with 1 μM PGE2 and analyzed by dot blot assay the levels of HHV-6 viral DNA. As shown in Fig. 9, HHV-6 DNA levels were increased in PGE2-treated cells compared with that of untreated cultures. PhosphoImager quantitation indicates a more than 2.5-fold enhancement in viral DNA level in PGE2-treated cultures. To
The transcriptional activity of the COX-2 promoter was capable of transactivating the COX-2 promoter (91). We investigated the role of IE proteins of HHV-6 as potential COX-2 promoter activators. Transcripts coding for IE1 and IE2 were demonstrated in HHV-6-infected monocytes with proteins bound to CRE and AP-1 supershifted with anti-CREB-1 and anti-c-JUN, respectively. By having determined that viral gene transcription is necessary for COX-2 promoter induction and that PGE2 is produced at early times (2–8 h) following HHV-6 infection of monocytes, we investigated the role of IE proteins of HHV-6 as potential COX-2 promoter activators. Transcripts coding for IE1 and IE2 were successfully detected in HHV-6-infected monocytes, at times when COX-2 protein levels were up-regulated (<8 h). By using expression vectors, we show that IE2, but not IE1, is capable of efficiently transactivating the COX-2 promoter. This result is in accordance with a previous report (84) demonstrating the activation of the human CD4 promoter by IE2 through a CRE. Interestingly, HCMV, another human betaherpesvirus, was reported to induce PGE2 secretion from smooth muscle cells (91). As is the case with HHV-6, IE proteins of HCMV were capable of transactivating the COX-2 promoter (91).

In the past few years, several studies (13, 14, 16–19, 50, 53, 56, 58–60) focused on COX-2 gene regulation, the limiting enzyme for PGE2 synthesis. Transcription factor-binding sites in the COX-2 promoter and their individual role as cis-acting elements involved in transcription are of particular interest. By using lipopolysaccharide-stimulated rodent macrophages and the THP-1 human monocytes cell line, Mestre et al. (55) have demonstrated redundancy in COX-2 promoter activation. Transcriptional shut down of COX-2 expression could not be accomplished by the targeting of a single transcription factor/promoter element (NFκB, nuclear factor-IL-6, or CRE). Previous studies (19, 50, 53, 56, 58–60, 92) also evidenced the importance of CRE and/or AP-1 element in mediating COX-2 transcription, particularly in the murine promoter, T cells, epithelial cells, and vascular cells. Regulation of COX-2 gene in human monocytes infected by viruses has yet to be studied. Our results show that both CRE and AP-1 elements are responsible for COX-2 promoter activation in HHV-6-infected monocytes. Interestingly, in human T lymphocytes, pNFAT/AP-1 element was key for COX-2 promoter induction following cellular activation (19). Our results clearly show that the pNFAT element is not implicated for the regulation of the COX-2 gene in monocytes infected with HHV-6. Furthermore, in HHV-6-infected Molt-3 T lymphocytes, the COX-2 promoter could be efficiently activated in an NFAT-independent manner (data not shown) suggesting that the nature of the stimuli is crucial in determining which transcription factors are associated with COX-2 gene activation.

Viruses elude immune detection through a diverse array of pathways. Altered antigen recognition through reduction of cell surface major histocompatibility complex class I is commonly exploited by viruses (for review see Ref. 93). Inhibition of apoptosis is also favored by several viruses to prevent premature cell death, allowing a more efficient synthesis of viral particles and spread of infection (93). Cytokines and other soluble factors are the messenger by which cells of the immune system can communicate between each other. The ability to modulate inflammatory mediators constitutes another efficient immunomodulatory strategy developed by viruses. In fact, several cytokines and other soluble factors such as eicosanoids are of particular importance in host defense and are frequently targeted by viruses. These include IL-10 by EBV, IL-12 by measles virus, IFN by adenovirus, and PGE2 by CMV, HIV, and HSV (26, 27, 94).

PGE2, an arachidonic acid metabolite, influences several inflammatory processes such as cytokine production, antibody formation, phagocytosis, and cell multiplication. PGE2 production has been observed following infection with several pathogens including viruses such as HSV-1, human cytomegalovirus (HCMV), HIV-1, and HTLV-1 (26, 27, 29, 94). It is worth noting that viruses with immunosuppressive properties such as CMV and HIV have been reported to induce PGE2 from monocytes, an event directly linked to impaired T cell proliferation (26, 27). Whether the ability of HHV-6 to inhibit T cell proliferation is linked to induction of PGE2 synthesis remains to be determined. Interestingly, treatment of mice with cyclooxygenase inhibitor antagonized vesicular stomatitis virus propagation, an effect likely attributable to the reduction of nitric oxide (NO) by prostaglandins (95). In fact, NO has been reported previously to play an important role in defense mechanisms against several viruses such as CMV, coxsackievirus, hepatitis B virus, and lymphohchoriomeningitis virus infection in mice (96–98).

In addition to its immunosuppressive activity on T cells and its ability to inhibit NO, PGE2 was also reported to positively modulate infections by CMV, HIV-1, and HSV (30–32). The results obtained in our study also suggest a positive effect of PGE2 on HHV-6 replication. Interestingly, a recent report (99) indicates that PGE2 synthesis impairment, through inhibition of the COX-2 enzyme, negatively affects CMV growth, suggesting that betaherpesviruses replication, such as that of HHV-6 and CMV, are influenced by eicosanoid production. Several studies (cited above) including this one suggest that PGE2 likely contributes to viral pathogenesis through several pathways. Detailed experiments using COX-knockout mice are warranted and should provide valuable information on the precise role of prostaglandins on viral growth and virally induced immunosuppression.

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