Herpes Simplex Virus Type 1-Mediated Induction of Human Immunodeficiency Virus Type 1 Provirus Correlates with Binding of Nuclear Proteins to the NF- κ B Enhancer and Leader Sequence

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Herpes simplex virus type 1 (HSV-1) infection induces expression of the human immunodeficiency virus type 1 (HIV-1) provirus in the chronically infected T-cell line ACH-2. The HSV-1-mediated induction correlates with the appearance of two NF- κ B-specific proteins of 55 and 85 kDa in the nucleus and with the binding of 50-kDa nuclear protein to the LBP-1 binding site of the untranslated leader sequence of the HIV-1 long terminal repeat. The HSV-1-induced LBP-1 binding protein, designated HLP-1, is present exclusively in HSV-1-infected, but not in phorbol-12-myristate-13-acetate- or tumor necrosis factor alpha-treated ACH-2 cells. Both the NF- κ B and LBP-1 target sequences, when inserted either alone or together 5' of a heterologous minimal promoter (thymidine kinase), confer inducibility by HSV-1 infection in a transient transfection assay. Thus, it appears that the HSV-1-mediated activation of HIV-1 provirus is brought about by the binding of both NF- κ B and HLP-1 specific proteins to two distinct regions of HIV-1 long terminal repeat.

The period between the initial infection with human immunodeficiency virus type 1 (HIV-1) and the clinical manifestation of AIDS disease can span several years. During this time, HIV-1 is present in infected cells as integrated proviral DNA, with little or no expression of viral components. The mechanism of transition from the latent, nonproductive HIV-1 infection cycle to a productive pathogenic infection is currently unknown. However, it has been shown that in chronically infected cell lines in vitro, HIV-1 expression can be activated by various stimuli such as mitogens, phorbol esters, and cytokines (reviewed in reference 34).

The signal transduction pathways by which these stimuli activate expression of HIV-1 provirus are still not clearly understood. Recently, it has been shown that the NF- κ B binding proteins that bind to the decameric sequence motif present in the enhancer element of the HIV-1 long terminal repeat (LTR) with concomitant induction of HIV-1 LTR expression play a major role in the activation of HIV-1 provirus by external stimuli (22, 27, 37). The transcription factor, NF- κ B, mediates transcription of a wide variety of cellular genes, coding for the immune, inflammatory, and acute-phase response proteins (2). This factor appears to be a heterodimer containing two DNA binding subunits of 50 and 65 kDa (3). The gene coding for the NF- κ B binding protein was recently identified, and it was shown that the amino-terminal half of its 105-kDa open reading frame encodes the 50-kDa subunit that shows a high homology to the *v-rel* oncogene and its cellular analogue, *c-rel* (8, 14, 20). While in B cells, NF- κ B is present in an active form in the nucleus (37), in the majority of other cells, it is present in the cytoplasm in an inactive form. Activation of NF- κ B generally requires dissociation of the inactive cytoplasmic NF- κ B-I κ B complex and translocation of the NF- κ B heterodimer to the nucleus (4). Activation may also involve the proteolytic cleavage that releases p50 from the 105-kDa precursor and the transcriptional activation of the NF- κ B gene (33).

The transcriptional activity of the HIV-1 LTR can also be stimulated by infection with several heterologous viruses, including those of the herpesvirus family that often accompany HIV-1 infection in AIDS patients (10, 15, 30, 36). The fact that several of these viruses use the NF- κ B factor to induce their own expression suggests that NF- κ B also plays a role in the up-regulation of HIV-1 infection by heterologous virus infections. We have analyzed the molecular mechanism by which herpes simplex virus type 1 (HSV-1) enhances expression of HIV-1 LTR and have shown, with others, that HSV-1-mediated transactivation of HIV-1 occurs at the transcriptional level (26). Using deletion analysis, we have localized the HSV-1-responsive element between –104 and –32 bp from the cap site and have shown that this 73-nucleotide-long fragment alone can confer HSV-1 inducibility in a transient expression assay (25). The presence of NF- κ B sites in the inducible element and the fact that HSV-1 infection stimulates the activation of NF- κ B binding proteins in HeLa cells (15) suggest a correlation between the binding of NF- κ B-specific proteins and the inducibility of the HIV LTR. In contrast, however, in Jurkat T cells expressing the HIV-1 *tat* gene, no single element conferring HSV-1 inducibility was identified and the inducibility of the HIV-1 LTR was not affected by mutations that abolished the NF- κ B-specific binding (28).

This study was initiated to examine the limiting factors that are involved in the HSV-1-mediated induction of HIV-1 expression in a chronically infected T-lymphocytic cell line, ACH-2. These cells contain a single copy of HIV-1 provirus which is expressed constitutively only at a very low level; however, its expression can be significantly enhanced by treatment with phorbol-12-myristate-13-acetate (PMA) or cytokines (34). Our results show that infection of ACH-2 cells with HSV-1 can also induce HIV-1 replication in these cells. The HSV-1-mediated induction correlates with the appearance of two NF- κ B binding proteins, p55 (55 kDa) and p85 (85 kDa), in the nucleus of HSV-1-infected cells and an HSV-1-induced 50-kDa protein, HSV-1-induced leader binding protein 1 (HLP-1), binding to the leader binding protein

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1 (LBP-1) region of the HIV-1 LTR. In a transient expression assay, both the NF- κ B and LBP-1 binding sites were able to confer inducibility by HSV-1. These results suggest that the HSV-1 activates HIV-1 provirus in ACH-2 cells by both NF- κ B-dependent and -independent transcription.

MATERIALS AND METHODS

Plasmids and oligonucleotides. The HIV-1 LTR chloramphenicol acetyltransferase (CAT) (pUIIR3) and κ B HIV-1 LTR CAT plasmids, obtained from C. Rosen and D. Baltimore, were described previously (25, 28).

Oligonucleotides were synthesized by a Millipore/Milligen DNA Synthesizer (Cyclone Plus) and gel purified on 8 M urea-polyacrylamide gels. The following oligonucleotides were used for DNA-binding studies or competitions:

κ B: TCAAGGGACTTTCGCTGGGGACTTTCCTCTCCTT

LBP-1: CTGGGTCTCTCTGGTTAGACCAGATCTGAGCCCTCTCCTT

HIP: TGCTACATATAAGCAGCTGCTTTTGCCTGTACTGGCCCTCTCCTT

mLBP CTTTGTCTCTCTTTT^{XX}TAGAA^{XX}AGATCTGAGCCCTCTCCTT

The underlined sequence was used for annealing of a primer, AAGGAGAGGG, and synthesis of the complementary strand. Mutations in the LBP-1 binding sites (17) within the mLBP oligonucleotide are marked. The probes for gel shift analyses were prepared by using Klenow polymerase (Life Technology, Inc.), [α - 32 P]dATP (Amersham), and the three unlabeled deoxynucleoside triphosphates (dNTPs). For the UV-cross-linking experiments, dTTP was substituted by 5-bromo-2'-deoxyuridine (BrdU) (Boehringer). Every labeling reaction was chased by all unlabeled dNTPs to ensure the double-strandedness of the probes.

Promoter constructs containing κ B and LBP-1 binding sites were generated by cloning of the respective oligonucleotides into pBLCAT2, which contains the -105 to +80 region of the *tk* gene promoter inserted in front of the CAT gene (23). p6tkCAT was generated by ligation of the κ B oligonucleotide into the *Sph*I site of pBLCAT2. p11tkCAT contains the LBP-1 oligonucleotide cloned into the blunt-ended *Xba*I site of pBLCAT2. p23tkCAT was prepared by insertion of the κ B oligonucleotide into the *Sph*I site of the p11tkCAT plasmid. Sequence and orientation were verified by sequencing with Sequenase (United States Biochemical [USB]) and M13 primers (USB). The orientation of the target sequences is identical to that in HIV-1 LTR.

Cells, viruses, and transfection assay. The A3.01 (12) and ACH-2 cells (9) were grown in RPMI medium supplemented with 10% fetal calf serum. HSV-1 stock was prepared by infection of Vero cells with HSV-1 strain WP at a multiplicity of infection (MOI) of 0.01 PFU per cell. The virus released from infected Vero cells by the freeze-thaw technique was used either directly or after purification on a sucrose gradient. No differences in the activation of HIV-1 or induction of DNA binding proteins were observed when unpurified (10^8 PFU/ml) or purified HSV-1 stock was used. Lysates from mock-infected Vero cells did not activate HIV-1 expression or binding of NF- κ B of HLP-1 proteins.

In the transfection assay, the CAT plasmid DNA (12 μ g) was introduced either by DEAE-dextran (240 μ g/ml) or by the electroporation technique into 2×10^7 cells. Twenty-four hours after transfection (electroporation), cells were divided into three parts: one part was treated with PMA (Sigma) (50 ng/ml), the second was infected with HSV-1 (MOI of 3), and the third was kept as untreated control. Sixteen hours after

PMA stimulation (or HSV-1 infection), cells were harvested, washed in phosphate-buffered saline, and frozen at -70°C . CAT analysis was performed as described previously (31).

Protein extracts and DNA binding analyses. Nuclear and cytoplasmic extracts were prepared as described by Stein et al. (38). In the binding reactions, approximately 5 μ g of protein extracts was incubated in 10 μ l of reaction mixture containing Tris (pH 7.6), 50 mM KCl, 5 mM MgCl_2 , 1 mM dithiothreitol, 0.1 mM EDTA, 6% glycerol, and 2 μ g of poly(dI-dC) (Boehringer) with the labeled oligonucleotide probe. The DNA-protein complexes were separated by electrophoresis in a 4% polyacrylamide gel in 0.4 $\times$ TBE buffer (35 mM Tris, 35 mM boric acid, 1 mM EDTA, pH 8.3).

UV-cross-linking was performed with the BrdU-substituted probe as described recently (41). Briefly, the binding and separation of the DNA-protein complex on nondenaturing gel was done as described above, and the gels were UV irradiated in situ for 20 min. The DNA-protein adducts were then eluted, and the eluates were analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Western immunoblot analysis and RT assay. Cells were lysed and proteins were separated as described recently (41). Separated proteins were then transferred to nitrocellulose filters and incubated with human anti-HIV-1 or -HSV-1 serum. Antigen-antibody complexes were detected by using ^{125}I -labeled protein A as described by Strebel et al. (39).

Virion-associated reverse transcriptase (RT) activity was measured by a modified method of Adachi et al. (1).

RESULTS

Activation of the endogenous HIV-1 provirus by HSV-1 infection. To determine whether HSV-1 infection can stimulate replication of HIV-1 provirus in the chronically infected T-cell line ACH-2, we infected cells with HSV-1 at an MOI of 3 and assessed HIV-1 replication by monitoring the levels of RT activity in the culture medium. Whereas we observed a sharp increase in RT activity at 24 h after HSV-1 infection in ACH-2 cells, no increase in RT activity was detected when media from mock-infected cells were used (data not shown).

The HSV-1-mediated induction of HIV-1 expression was further analyzed on the protein level by Western blot analysis (Fig. 1A). In ACH-2 cells, relative levels of HIV-1 proteins were markedly increased at 24 h after HSV-1 infection, while HSV-1-specific proteins were detected as early as 8 h after infection (Fig. 1B).

Analysis of NF- κ B binding proteins induced by HSV-1. To determine whether infection of ACH-2 cells with HSV-1 results in the induction of specific nuclear proteins that can bind to the regulatory elements in the HIV-1 LTR, we examined by gel retardation assay the binding of nuclear proteins from HSV-1-infected ACH-2 cells to the fragment containing the enhancer element of HIV-1 LTR (*Sca*I [-140] to *Dde*I [-40] fragment). Extracts from HSV-1-infected cells showed the presence of a new DNA-protein complex. Migration of this complex was identical to that of the complexes induced in these cells by treatment with PMA and tumor necrosis factor alpha (TNF- α), and its formation was prevented by addition of the oligonucleotide representing the HIV-1 κ B enhancer element, but not by its mutated analog that lost NF- κ B binding (data not shown), indicating that the HSV-1 infection of ACH-2 cell line resulted in the induction of NF- κ B binding proteins.

It has previously been shown that the κ B enhancer is a

FIG. 1. Western blot analysis of HIV-1- and HSV-1-specific proteins in HSV-1-infected ACH-2 cells. Cells lysates were prepared from uninfected cells and from HSV-1-infected cells collected at 3, 8, 24, 48, and 72 h postinfection. Proteins (50 μ g) were separated on an SDS-10% polyacrylamide gels, transferred to nitrocellulose filters, and incubated with anti-HIV-1 antiserum (A) or anti-HSV-1 antiserum (B). The positions of the *gag* proteins, p24 and p55, and of the envelope glycoprotein, gp120, are indicated to the left of panel A, and those of prestained protein molecular weight standards ($\times 10^3$) (Life Technologies, Inc.) are indicated on the left of panel B.

target for the binding of several proteins of the Rel family (5, 24). To determine which of the NF- κ B binding proteins are induced by HSV-1 in ACH-2 cells, we incubated nuclear extracts from HSV-1-infected and uninfected ACH-2 cells with the photoactivatable BrdU-substituted NF- κ B probe (see Materials and Methods). The protein-DNA complexes were separated on a nondenaturing gel (Fig. 2A) and irradiated in situ, and the eluted DNA-protein adducts were resolved by SDS-PAGE. HSV-1 infection of ACH-2 cells

FIG. 2. UV-cross-linking analysis of protein-DNA complexes formed between the NF- κ B sequence and nuclear extracts from HSV-1-infected ACH-2 cells. Nuclear extracts prepared from HSV-1-infected cells at 3 or 8 h postinfection were incubated with a BrdU-substituted NF- κ B probe. DNA-protein complexes were resolved on a 4% polyacrylamide gel (A). Gels were UV-cross-linked in situ, bands representing protein-DNA complexes were eluted, and the nucleoprotein adducts were analyzed on an SDS-10% polyacrylamide gel (B). Positions of prestained protein molecular weight standards ($\times 10^3$) are indicated on the right (B). The numbers above the lanes indicate hours after HSV-1 infection.

FIG. 3. Effect of CHX treatment on NF- κ B binding activity in HSV-1-infected or PMA-stimulated ACH-2 cells. Nuclear extracts were prepared from untreated cells (lane 1), HSV-1-infected cells (lane 2), or HSV-1-infected cells treated with CHX for the indicated times (hours) (lanes 3, 4, and 5). PMA-stimulated cells (lanes 6 and 8) or cells treated with CHX (lanes 6 and 7) were used as controls. The total length of infection in all cases was 7 h. Extracts were incubated with the 32 P-labeled NF- κ B probe (see Materials and Methods), and DNA-protein complexes were resolved on a 4% polyacrylamide gel.

induced binding of two NF- κ B specific proteins of 55 and 85 kDa (Fig. 2B). We assume that the smaller protein may represent an alternatively processed form of NF- κ B p50 (14), and the larger protein the 85-kDa *c-rel* proto-oncogene (32); however, additional experiments are in progress to determine the identification of these proteins. Identical NF- κ B proteins were induced in the HSV-1-infected, HIV-1-negative parental T-lymphocytic cell line A3.01 (data not shown).

To correlate the induction of NF- κ B binding proteins with the time course of the HSV-1 replicative cycle in T cells and to determine whether protein synthesis is required for NF- κ B induction, we treated ACH-2 cells with cycloheximide (CHX) at different times after HSV-1 infection. NF- κ B binding proteins appeared in HSV-1-infected ACH-2 cells at 4 to 5 h after infection (data not shown). Addition of CHX at 1 or 2 h after infection (Fig. 3, lanes 5 and 3, respectively) inhibited the appearance of NF- κ B binding activity in nuclei of HSV-1-infected cells. However, when CHX was added at 4 h after infection (Fig. 3, lane 4), inhibition was only marginal. Treatment of cells with CHX alone did not stimulate the binding of NF- κ B. In contrast, PMA-induced NF- κ B activity was not affected by CHX treatment. These results show that the HSV-1-mediated activation of NF- κ B requires protein synthesis, suggesting that the synthesis of viral

FIG. 4. Binding of a specific nuclear protein from HSV-1 ACH-2 cells to the sequence (−40 to +36) of the HIV-1 LTR. Nuclear extracts were prepared from untreated cells, cells treated with TNF- α , PMA (lanes 4 and 5), or bryostatin-1 (BRY-1) for 2 or 8 h, or cells infected with HSV-1 for 2 or 8 h. The DNA-protein complexes were resolved by a gel retardation assay as described in the legend to Fig. 2. The HSV-1-inducible protein-DNA complex is indicated by an arrow.

proteins is required either for the activation of NF- κ B gene expression or for its dissociation from an inactive NF- κ B-I κ B complex. The present study, however, does not exclude completely the possibility that the synthesis of cellular proteins is also required for the activation of NF- κ B binding. We have previously shown that the immediate-early genes of HSV-1 are involved in the activation of the HIV-1 LTR (25). However, it is still not clear whether these genes also mediate the induction of NF- κ B binding activity in the nucleus in HSV-1-infected cells, since at the MOI used for the activation of HIV-1 provirus, expression of both immediate-early (IE110, IE175) and late genes (VP16) could be detected at the time of appearance of NF- κ B activity in the nucleus (data not shown).

Infection with HSV-1 induces binding of a novel protein to the HIV-1 leader sequence. Based on several reports in which HIV-1 LTR CAT deletion mutants were used, the inducibility of the HIV-1 LTR by cytomegalovirus and other viruses depends on the presence of sequences downstream from the transcription initiation site (6, 7). To determine whether HSV-1 induces nuclear proteins that can interact with this region, we examined binding of nuclear proteins from HSV-1-infected cells to the fragment of the HIV-1 LTR representing the sequence between the TATA box (*Dde*I [−40]) and the end of the TAR region (*Sac*I [+36]). Formation of a new inducible protein-DNA complex (Fig. 4) was observed in cells infected with HSV-1 but not in the cells induced with TNF- α , PMA, or bryostatin-1, which can also activate the HIV-1 provirus (21, 41). Since this region contains, in addition to the TATA box and TAR regions, the binding site for the HIV-1 initiator protein (HIP) (18) and three binding sites for the LBP-1 protein (18, 43), we used oligonucleotide probes corresponding to either the HIP region (−25 to +10) or to the LBP-1 binding region (−3 to +36) to define more closely the binding site for this new protein. We found that the HSV-1-inducible protein(s) herein called HLP-1 (HSV-1-induced leader binding protein 1) interacts with the LBP-1 sequence (Fig. 5A), but not with the HIP sequence (data not shown).

The time course of induction of the HLP-1 binding activity in HSV-1-infected A3.01 cells is shown in Fig. 5A. Infection

FIG. 5. Time course of induction of HLP-1 binding activity in HSV-1-infected A3.01 cells. (A) Nuclear (nuc.) and cytoplasmic (cyt.) extracts were prepared from cells infected with HSV-1 for 5, 10, 15, or 20 h or untreated controls and incubated with the 32 P-labeled LBP-1 oligonucleotide probe (see Materials and Methods) (−3 to +36). DNA-protein complexes were resolved as described in the legend to Fig. 3. (B) Competition (compet.) of HLP-1 binding by oligonucleotides representing LBP-1, HIP, NF- κ B, and mLBP binding sites, respectively. The oligonucleotides (described in Materials and Methods) were added in 100-fold excess to the nuclear extracts before the addition of the radioactive LBP-1 probe.

resulted in both the appearance of a new slowly moving complex (marked a in Fig. 5A) and enhancement of binding of a fast-moving complex (b). Complex b is constitutively present in the nucleus and cytoplasm of uninfected A3.01 cells as well as in HeLa and Namalwa cells (see also Fig. 6). The level of the cytoplasmic b complex was greatly enhanced at later times after HSV-1 infection, when it had the mobility of a doublet. The HLP-1 binding activity (complex a) appeared in the nucleus of A3.01 cells by 5 h postinfection (seen upon a prolonged exposure), and its level gradually increased with the progression of infection. In cytoplasm, the HLP-1 binding activity could be detected as early as at 5 h postinfection. The same pattern of HLP-1 binding was also observed in HSV-1-infected Jurkat, ACH-2, and Namalwa cells (data not shown), suggesting that this binding activity is associated with HSV-1 infection, but it is not cell-type specific. The specificity of HLP-1 binding to the LBP-1 site was confirmed by competition experiments. Binding of HLP-1 to the radioactive LBP-1 probe in a gel retardation assay (Fig. 5B) could be inhibited by the addition of a 100-fold excess of the unlabeled probe, but not by the same excess of oligonucleotides representing NF- κ B or HIP binding sites. Also, the LBP-1 oligonucleotide mutated in all three LBP-1 binding sites (17) was not able to block HLP-1 binding at this concentration.

It has been shown that the NF- κ B proteins p50 and p65 are present in the cytoplasm of the majority of cell types as an inactive NF- κ B-I κ B complex. To determine whether the HLP-1 protein is also present in the cytoplasm of uninfected cells in an inactive form, we treated the cytoplasmic extracts from uninfected A3.01 cells with several detergents (Nonidet P-40, sodium deoxycholate, CHAPSO); however, no induc-

FIG. 6. Effect of CHX treatment on HLP-1 binding activity in HSV-1-infected A3.01 cells. Nuclear and cytoplasmic extracts were prepared from untreated cells (lane 1), from cells infected with HSV-1 for 7 h (lane 2), and from HSV-1-infected cells treated with CHX for the indicated times (hours) (lanes 3, 4, and 5). Extracts from cells treated with PMA (lanes 6 to 8) and CHX (lanes 6 and 7) served as controls. The binding was analyzed as described in the legend to Fig. 5.

tion of HLP-1 binding activity was detected. Similar treatment of cytoplasmic extracts released the NF- κ B binding activity (data not shown).

To assess whether protein synthesis is required for the HSV-1-mediated induction of HLP-1 binding, A3.01 cells were infected with HSV-1 and then incubated for different lengths of time with CHX. Cells were collected 7 h after HSV-1 infection, and the nuclear and cytoplasmic extracts were analyzed for HLP-1 binding activity (Fig. 6). Addition of CHX as late as 4 h after infection completely abolished the induction of HLP-1 binding activity in the nucleus (Fig. 4), while low levels of HLP-1 binding activity could be detected in the cytoplasm, suggesting that the HLP-1 protein starts to appear in the cytoplasm 4 h after infection and is later translocated into the nucleus.

To determine the size of the HLP-1 protein, we incubated nuclear and cytoplasmic extracts from HSV-1-infected ACH-2 cells with the BrdU-substituted LBP-1 probe. After separation of protein-DNA complexes in a nondenaturing gel and in situ UV-cross-linking, nucleoprotein adducts were analyzed by SDS-PAGE (Fig. 7). Comparison of protein complexes a and b (Fig. 5) revealed a 50-kDa nucleoprotein adduct to be the major component of slowly migrating complex a. This nucleoprotein complex was present in both the nucleus and cytoplasm. In contrast, complex b is formed by several different proteins that can be seen only upon a long exposure (Fig. 7). On the basis of this experiment, we conclude that the HLP-1 protein is not identical to the previously identified LBP-1 protein of 61 to 64 kDa (43).

Functional analysis of HSV-1-inducible proteins. To deter-

FIG. 7. UV-cross-linking of proteins to the LBP-1 probe. Nuclear (lanes 1 and 2) and cytoplasmic (lanes 3 and 4) extracts from HSV-1-infected ACH-2 cells were incubated with the BrdU-substituted LBP-1 probe, and nucleoprotein complexes were separated by gel retardation and cross-linked in situ. Complexes a (lanes 1 and 3) and b (lanes 2 and 4) (Fig. 5) were excised, and nucleoprotein adducts were analyzed on an SDS-10% polyacrylamide gel. The molecular size markers ($\times 10^3$) are indicated on the right.

mine the possible function of NF- κ B and HLP-1 proteins during HSV-1-mediated induction of the HIV-1 LTR, we examined the HSV-1-mediated activation of the HIV-1 LTR CAT reporter plasmid in A3.01 cell lines by a transient expression assay (Fig. 8). As a control for induction, we also stimulated these cells with PMA, which induces several NF- κ B binding proteins in these cells (41), but not the HLP-1 protein. Although the basal level of CAT activity in these cells was very low, stimulation with PMA resulted in a threefold increase of CAT levels, while HSV-1 infection increased the enzyme levels by ninefold. In the A3.01 cells transfected with the NF- κ B mutant HIV-1 LTR CAT plasmid (mutation in NF- κ B sites) (28), treatment with PMA did not increase the level of CAT activity (Fig. 8), while HSV-1 infection increased the enzymatic activity by threefold.

FIG. 8. Activation of promoter in HIV-1 LTR CAT plasmid by HSV-1 infection in A3.01 cells. Cells were transfected with HIV-1 LTR-CAT or m κ BHIV-1 LTR CAT plasmids as described in Materials and Methods, and 24 h after transfection, cells were left untreated (■), treated with PMA for 16 h (▨), or infected with HSV-1 at an MOI of 3 for 16 h (□). CAT activities are shown as the fold increase relative to CAT activities of untreated cells. A representative of three independent transfections is shown. The variability between the individual experiments was about 15%.

FIG. 9. The NF- κ B and HLP-1 binding sites confer HSV-1-mediated inducibility to a heterologous promoter. The p6tkCAT, p11tkCAT, and p23tkCAT plasmids, as well as the parental vector, pBLCAT2, were transfected into A3.01 cells, and 24 h after transfection cells were either treated with PMA (▨) or infected with HSV-1 at an MOI of 3 for 16 h (□). CAT activities are shown as a percentage of conversion. Numbers under each lane represent an average value of three independent experiments and indicate the fold increase of CAT activity compared with the activity of untreated cells (■).

Therefore, in addition to the NF- κ B enhancer, another *cis* element of the HIV-1 LTR contributes to the HSV-1-mediated induction.

Since we showed that HSV-1 infection induces both NF- κ B and HLP-1 binding activities in the nucleus, we examined the relative contribution of these sequences to the inducibility of the HIV-1 LTR by HSV-1 infection. We cloned the NF- κ B and LBP-1 target sequences either alone or in combination into the pBLCAT2 vector (23), which carries the CAT gene under control of the thymidine kinase (*tk*) minimal promoter. The plasmids containing two NF- κ B binding sites (p6tkCAT), three LBP-1 binding sites (p11tkCAT), or both the NF- κ B and the LBP-1 sites (p23tkCAT) and the parental vector, pBLCAT2, were transfected into A3.01 cells, and transfected cells were either stimulated with PMA or infected with HSV-1. Expression of all transfected plasmids was stimulated by HSV-1 infection, while only the plasmids that contained the NF- κ B enhancer (p6tkCAT and p23tkCAT) were stimulated by PMA (Fig. 9). The promoter activities of both p6tkCAT containing NF- κ B sites and p11tkCAT containing LBP-1 sites were activated by HSV-1 infection more effectively (10-fold and 7-fold increase, respectively) than the parental pBLCAT2 vector (4-fold induction), indicating that either site can confer inducibility by HSV-1. The p23tkCAT plasmid containing both the NF- κ B and HLP-1 binding sites was, however,

induced by HSV-1 most efficiently, and the levels of CAT activity were about 25-fold higher in HSV-1-infected cells than in uninfected cells. These results suggest a cooperative effect between NF- κ B and HLP-1 proteins.

DISCUSSION

In this study, we showed that the HSV-1-mediated activation of HIV-1 provirus in T cells correlated with binding of regulatory proteins to at least two *cis*-acting elements of the HIV-1 LTR: the NF- κ B decamers and the LBP-1 sites of the leader sequence. It was shown that the activation of the HIV-1 LTR and of the integrated HIV-1 provirus by a number of external stimuli proceeds through up-regulation of NF- κ B-mediated transcription. The stimulation of T cells and monocytes with PMA or TNF- α induces binding of about five different polypeptides, including NF- κ B protein p50 and *c-rel* (5, 24, 41). By contrast, HSV-1 infection of T-lymphocytic cell lines A3.01 and ACH-2 activated only binding of the p55 and p85 polypeptides, which we assume correspond to the alternatively processed p50 subunits of NF- κ B and *c-rel*, respectively (14, 20). However, additional experiments with specific antisera must be done to confirm the identity of these proteins. Both the *c-rel* and the NF- κ B complexes were previously shown to activate NF- κ B-mediated transcription (2, 16). The nuclear form of the NF- κ B complex contains both the 50- and 65-kDa protein subunits that bind DNA as a heterodimer; the presence of the 65-kDa polypeptide seems to be essential for the inducibility. Since p65 can also bind the NF- κ B decamer (29, 35), its absence in nuclear extracts of HSV-1-infected cells detected by UV-cross-linking is surprising. As reported by Urban et al. (40), the inability to detect p65 can be a consequence of the weak reactivity of p65 with the BrdU-substituted probe or reflects a lower binding preference of p65 for the NF- κ B motif present in the HIV-1 LTR. Further experiments are in progress to address this question.

The appearance of NF- κ B proteins in the nucleus of HSV-1-infected cells required protein synthesis, while this was not necessary for the PMA-mediated activation of NF- κ B binding. We have previously shown (26) that expression of the HSV-1 viral genome was essential for the activation of integrated HIV-1 LTR in Vero cells. Whether the activation of NF- κ B binding by HSV-1 gene products involves dissociation of the inactive cytoplasmic NF- κ B-I κ B complex and transport of p65-p50 heterodimer to the nucleus or other forms of activation, such as up-regulation of transcription of the respective genes, is not known. It is also not completely clear at present which HSV-1-encoded gene product is required for the induction of NF- κ B binding activity in the nucleus. We (25) and others (13, 28) have previously shown that the expression of the HSV-1 immediate-early genes ICP0 and ICP4 was required for the activation of the HIV-1 LTR in a transient expression assay. We could not detect any increase in NF- κ B binding activity in A3.01 cells transfected with the expression plasmids coding for either the ICP0 or ICP4 protein (42). At the multiplicity of HSV-1 infection used, transcription of both the early (*tk*) and late (VP16) HSV-1 genes had already occurred at times (4 to 5 h postinfection) when the NF- κ B binding activity was first detected in the nucleus. However, inhibition of HSV-1 replication by acyclovir substantially decreased induction of NF- κ B binding in HSV-1-infected cells (42), suggesting that the expression of the early HSV-1 genes is not sufficient for the induction of NF- κ B binding activity.

In the present study, we also showed that mutation in both

NF- κ B sites of the HIV-1 LTR did not completely abolish inducibility by HSV-1 infection and that HSV-1 infection induces binding of a new protein designated HLP-1 to the LBP-1 site in the untranslated leader region of the HIV-1 LTR. It is not without interest that this region is also critical for the cytomegalovirus-mediated induction of the HIV-1 LTR (6, 7). The induction of HLP-1 binding activity was completely blocked in the absence of protein synthesis, and HLP-1 appeared in the nucleus at about the same time as NF- κ B binding activity (at about 5 h). It was previously shown by others that this region also binds constitutively expressed LBP-1 protein (43). Addition of purified LBP-1 protein to an in vitro transcription system inhibited HIV-1 LTR-directed transcription (19). We have shown that mutations in the LBP-1 binding sites that prevented LBP-1 binding (17) decreased also the binding of HLP-1 protein, suggesting that both of these proteins recognize similar DNA sequences. We believe, however, that HLP-1 is not identical to LBP-1 for three reasons: (i) HLP-1 is present exclusively in HSV-1-infected human T and B cells and not in uninfected cells or PMA- or TNF- α -stimulated cells; (ii) the results of the transfection experiments with the p11tkCAT and p23tkCAT plasmids have shown that the LBP-1 site acts as an inducible enhancer specifically in HSV-1-infected cells, but not in uninfected controls; and (iii) the LBP-1 protein was shown to be 61 to 64 kDa, while the results of UV-cross-linking analysis indicate that the major protein in HSV-1-infected cells that binds to this region is 50 kDa. A question that remains is whether the HLP-1 protein is of cellular or viral origin. We and others have previously shown (25, 28) that ICP0 and ICP4 can activate the HIV-1 LTR in a transient expression assay. Nevertheless, the LBP-1 probe does not contain the ICP-4 binding site (ATCGTC) (11), and transfection with ICP-4-expressing vector did not induce HLP-1 binding activity (42). It will be interesting to examine whether HLP-1 is induced also by infection with other transactivating herpesviruses such as cytomegalovirus and human herpesvirus 6 and whether it can transactivate other genes in addition to the HIV-1 LTR.

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