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Differential Contribution of Herpes Simplex Virus Type 1 Gene Products and Cellular Factors to the Activation of Human Immunodeficiency Virus Type 1 Provirus

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We have previously reported that infection with herpes simplex virus type 1 (HSV-1) activates expression of the human immunodeficiency virus type 1 (HIV-1) provirus in T cells. Activation of the HIV-1 provirus correlated with the activation of binding of 55- and 85-kDa proteins to the κB enhancer and binding of the 50-kDa HLP-1 protein to the LBP-1 sequences of the HIV-1 long terminal repeat. Further examination of this system has shown that the inhibition of HSV-1 replication by the antiviral drug acyclovir does not inhibit HSV-1-mediated induction of HIV-1 provirus. Surprisingly, the NF-κB and HLP-1 binding activities were substantially inhibited in acyclovir-treated cells. In the transient-transfection assay, ICP0, but not ICP4, activated the HIV-1 long terminal repeat promoter region and the effect of ICP0 was greatly enhanced in the presence of the NF-κB binding proteins, suggesting that induction of the HIV-1 provirus involves cooperation between the HSV-1-activated cellular factor, NF-κB, and the virus-encoded transactivator, ICP0.

Expression of human immunodeficiency virus type 1 (HIV-1) in chronically infected cells can be stimulated by several agents, including cytokines, phorbol esters, mitogens, and infection with heterologous viruses (5, 14, 15). Members of the herpesvirus family have been shown to stimulate transcription driven by the HIV-1 long terminal repeat (LTR), and we have recently shown that infection with herpes simplex virus type 1 (HSV-1) induces HIV-1 expression in the chronically infected T-lymphocytic cell line ACH-2 (20). The activation of provirus transcription by HSV-1 correlates with binding of the 55- and 85-kDa proteins to the NF-κB binding site and with binding of the 50-kDa protein (HLP-1) to the LBP-1 site in the leader sequence of the HIV-1 LTR (9, 10, 20). In transient-expression assays using the HIV-1 LTR CAT plasmids, the herpesvirus-encoded transactivators ICP0 and ICP4 were also shown to stimulate the HIV-1 LTR-directed transcription (11–14, 16, 22). However, the activation by the immediate-early proteins (IE), ICP0 and ICP4, has not yet been associated with binding to any specific DNA sequence in the HIV-1 LTR. In contrast to the transient-expression assay, the overexpression of ICP0 or ICP4 is able neither to activate transcription of the integrated HIV-1 provirus nor to induce binding of NF-κB and HLP-1 proteins (21). Replication of HSV-1 can be blocked by the nucleoside analog acyclovir (ACV), which is effectively incorporated into the newly formed viral DNA with consequent premature termination of viral DNA synthesis. It was shown that, in the presence of ACV, expression of the IE and the early HSV-1 genes is not altered but expression of the late genes is inhibited. In this report, we have examined whether the treatment of HSV-1-infected cells with ACV inhibits activation of the HIV-1 provirus.

The induction of the HIV-1 provirus was studied in a T-lymphocytic cell line, ACH-2 (4), containing a single copy of the HIV-1 provirus. Activation of HIV-1 expression in these cells by HSV-1 infection was described recently (20). The ACH-2 cells were infected with HSV-1 at a multiplicity of infection (MOI) of 1 to 3 in the presence (5 or 30 μM) or the absence of ACV, and the HIV-1-specific proteins were analyzed by Western blot (immunoblot) analysis at 16 and 40 h postinfection (Fig. 1). Surprisingly, the levels of HIV-1-specific proteins in the presence and absence of ACV were about the same. Treatment of the ACH-2 cells with ACV alone did not alter HIV-1 expression (data not shown).

Next, we examined the effect of ACV treatment on HIV-1 transcription in ACH-2 cells infected with HSV-1. Cells were infected with HSV-1 at an MOI of 3 in the absence or presence of ACV (5 and 30 μM), and the levels of HIV-1 transcripts at 4 and 8 h postinfection were determined by S1 mapping using the HIV-1 antisense riboprobe spanning the first 80 bases of all HIV-1 transcripts (Fig. 2). While no stimulation of the HIV-1 transcription could be detected 4 h after HSV-1 infection, at 8 h postinfection the levels of HIV-1 transcripts were increased in HSV-1-infected cells and the relative levels of HIV-1 transcripts present in ACV-treated cells were higher than in the controls. The increase was proportional to the concentration of ACV used (Fig. 2, lanes 7 and 8). Treatment of ACH-2 cells with ACV alone did not result in any stimulation of HIV-1 transcription (Fig. 2, lane 9). The ACV treatment also had a marked effect on HSV-1-stimulated production of HIV-1. In HSV-1-infected ACH-2 cells, virus production was markedly increased at 24 h postinfection but then declined due to the cytopathic effect of HSV-1. However, in the presence of ACV, HIV-1 virions were effectively synthesized even at 96 h postinduction (data not shown). Thus, the ACV-induced block in HSV-1 replication and cell death led to prolonged production of HIV-1.

To determine the effectiveness of ACV treatment on the expression of HSV-1 genes in T lymphocytes, we have analyzed the expression of three HSV-1 genes, ICP0, tk, and VP16, which represent the three defined classes of the immediate-early, early, and late HSV-1 genes, respectively. The ACH-2 cells were infected with HSV-1 in the presence or absence of ACV, and at the indicated times, cells were

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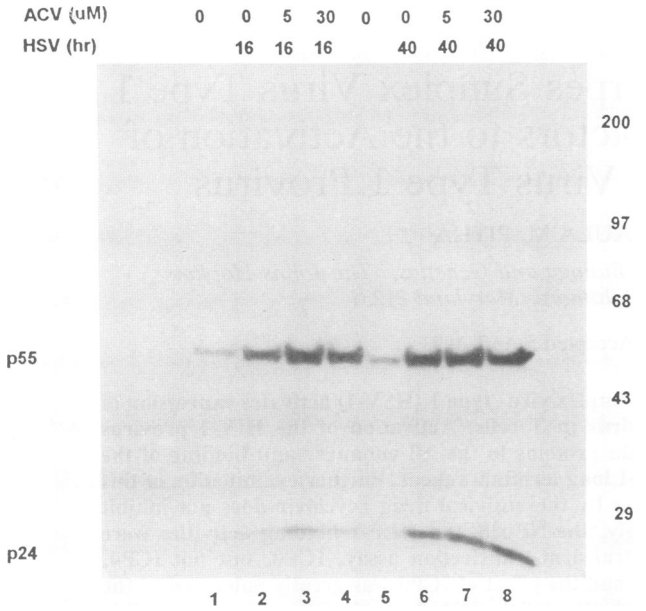

FIG. 1. Western blot analysis of the HIV-1 proteins. One hundred micrograms of protein lysates was loaded on sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis and transferred on nitrocellulose membrane, and the HIV-1-specific proteins were detected with the antiserum from an AIDS patient. Cells were infected with HSV-1 at an MOI of 3 for the times shown in the absence or presence of various concentrations of ACV (Burroughs-Wellcome, Research Triangle Park, N.C.), as indicated for lanes 2 to 4 and 6 to 8. Lanes 1 and 5 represent lysates prepared from mock-infected cells. Positions of Gag proteins p55 and p24 are indicated.

collected and RNA was analyzed by Northern (RNA) hybridization. Figure 3 shows that the relative levels of ICP0 mRNA are only slightly lower in ACV-treated cells, whereas the levels of tk transcripts were not significantly affected by the presence of ACV, implying that ACV does not inhibit transcription of the immediate-early and early HSV-1 genes. In contrast, the presence of ACV had a profound effect on transcription of the late genes, and even 5 μM ACV significantly reduced levels of VP16 mRNA. The observed variation in the levels of ICP0 and a decrease in VP16 levels are not due to the difference in RNA loading, since the ethidium bromide staining shows an equal amount of RNA in all samples. The observed inhibition of ICP0 mRNA at 7 h postinfection is probably a consequence of lower levels of VP16 that is an effective transactivator of ICP0 gene expression. These results suggest that activation of the latent HIV-1 provirus by HSV-1 does not require viral replication and expression of the late viral genes.

Previously, we have shown that the HSV-1-mediated activation of the HIV-1 provirus correlated with the induction of binding of nuclear proteins to the κB enhancer and the leader region of HIV-1 LTR (20). To determine whether HSV-1-mediated induction of the NF-κB and HLP-1 proteins is altered in ACV-treated cells, we used the electrophoretic mobility shift assay to analyze the binding of nuclear extracts from ACV-treated and non-ACV-treated, HSV-1-infected ACH-2 cells to NF-κB and LBP-1 probes (Fig. 4A and B). The results show that ACV significantly inhibits the HSV-1-mediated induction of NF-κB and HLP-1 proteins. However, even when high levels of ACV were

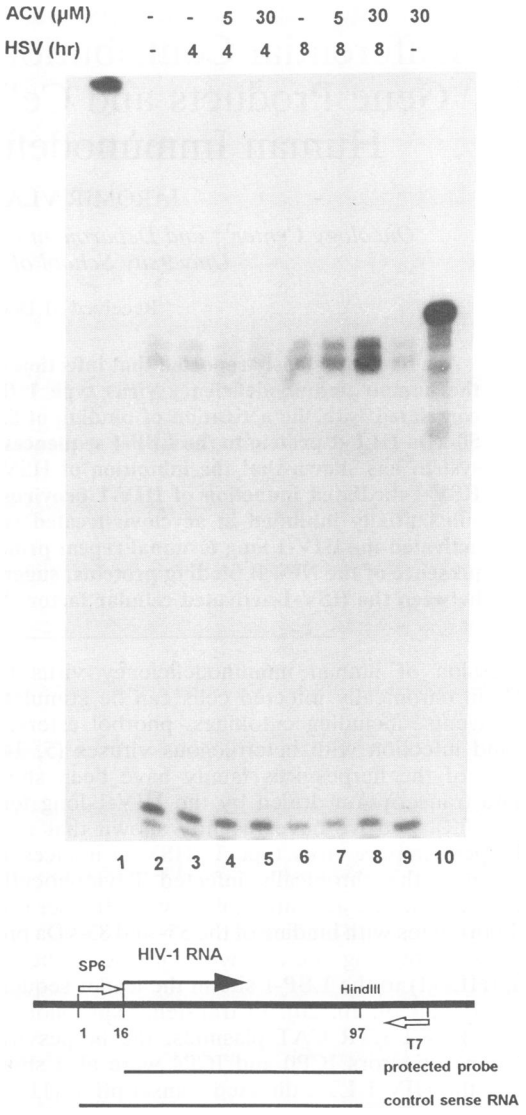

FIG. 2. Analysis of HIV-1 RNA levels in HSV-1-infected ACH-2 cells. ACH-2 cells were infected with HSV-1 in the absence or presence of the indicated concentrations of ACV. RNA was isolated at 4 and 8 h postinfection by the acid phenol method (3). Two micrograms of the total RNA was hybridized with 4×10^5 cpm of labeled riboprobe (described below) in 40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.5)–1 mM EDTA–0.4 M NaCl–80% formamide for 16 h at 50°C. S1 nuclease was added in 0.28 M NaCl–50 mM sodium acetate (pH 4.5)–4.5 mM $ZnSO_4$, and the reaction proceeded for 40 min at 37°C. After precipitation, the RNA was electrophoresed through an 8% sequencing gel. The antisense riboprobe is shown in lane 1. Plasmid p4TAR30 contains sequences *Rsa*I (−4) to *Hin*dIII (+80) of the HIV-1 LTR inserted between the *Eco*RI and *Hin*dIII sites of pGem4Z (Promega, Madison, Wis.). The antisense riboprobe was prepared from p4TAR30 linearized with *Pvu*II by in vitro transcription in the presence of [^{32}P]GTP (New England Nuclear, Boston, Mass.) using T7 RNA polymerase (Boehringer, Indianapolis, Ind.) (bottom panel). The sense RNA used as a standard in lane 10 was prepared by in vitro transcription of p4TAR30 linearized with *Hin*dIII with SP6 RNA polymerase (Boehringer). This RNA gives a protected fragment larger than HIV-1 transcripts due to its complementarity with the antisense probe in the region of the SP6 promoter.

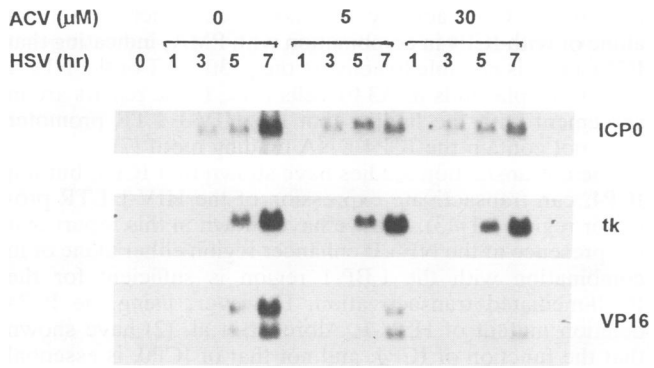

FIG. 3. Kinetics of mRNA levels of ICP0, thymidine kinase (tk), and VP16 during infection of ACH-2 cells with HSV-1. Ten micrograms of the total RNA was electrophoresed through 1% agarose containing formaldehyde, blotted on the nitrocellulose membrane, and hybridized with the probes specific for the respective genes. The concentration of ACV used during infection is shown on top.

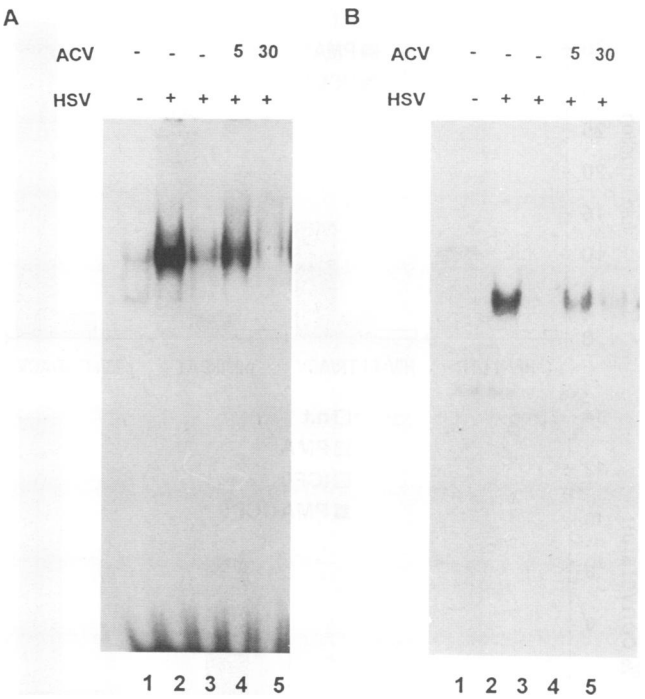

FIG. 4. Electrophoretic gel-shift analysis of the proteins binding to the NF-κB (A) and LBP-1 (B) probes. Nuclear extracts were prepared by the freeze-thaw method (19) from uninfected cells (lanes 1) or cells infected with HSV-1 for 12 h in the absence or presence of 30 μM ACV (lanes 2 to 5). Approximately 3 μg of protein was incubated with the labeled probe in 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.6–5 mM $MgCl_2$–50 mM KCl–6% glycerol–2 μg of poly(dI-dC) for 10 min at 22°C and then electrophoresed through 4% nondenaturing acrylamide gel. The NF-κB probe contains the NF-κB sequence from the HIV-1 LTR as described previously (20), and the LBP-1 probe contains the sequences from the leader region of the HIV-1 LTR (20). Nuclear extracts in lanes 3 were incubated with a 100-fold molar excess of the unlabeled probes. Both radioactive probes were prepared by fill-in reaction using Klenow polymerase (Life Technologies, Grand Island, N.Y.).

used, the binding was not completely abolished. We have previously shown that activation of HIV-1 provirus was not proportionally related to the levels of NF-κB binding proteins in the nucleus and that even very low levels of the NF-κB-specific protein binding are sufficient to activate the latent HIV-1 provirus (19).

To determine whether the levels of NF-κB and HLP-1 proteins present in ACV-treated cells are sufficient to activate the HIV-1 provirus, we measured the activation of the reporter plasmid, p23tkCAT, in the HSV-1-infected and ACV-treated A3.01 cells. This plasmid, which contains the NF-κB and HLP-1 binding sites in front of the tk promoter, is effectively induced in HSV-1-infected A3.01 cells (20). p23tkCAT was transfected into ACV-treated cells or untreated controls, and 24 h after transfection, cells were either treated with phorbol 12-myristate 13-acetate (PMA) or infected with HSV-1. The HIV-1 LTR CAT plasmid was transfected for comparison. The results (Fig. 5A) show that, while the constitutive expression of these plasmids was only marginal and PMA treatment increased their expression about threefold, HSV-1 infection increased expression of p23tkCAT by 25-fold. The inhibition of HSV-1 replication in ACV-treated cells, with a concomitant decrease in the levels of NF-κB and HLP-1 binding, did not abolish expression of the CAT gene but enhanced it. Similar results were obtained with the HIV-1 LTR CAT reporter plasmid. Enhancement of the HSV-1-mediated expression of HIV-1 LTR in the presence of ACV indicates that products encoded by the IE or the early HSV-1 genes may play a significant role in the activation. The best candidates are the ICP0 and ICP4 immediate-early genes. Neither the relative levels of ICP0 and ICP4 mRNAs (Fig. 3) nor the activities of their promoter regions (Fig. 5C) are significantly affected by ACV treatment.

The ability of ICP0 and/or ICP4 to activate transcription driven by the HIV-1 LTR in a transient-expression assay has been documented previously (11–14). In contrast, the overexpression of ICP0 or ICP4 can induce neither the HIV-1 provirus nor the NF-κB and HLP-1 binding proteins in ACH-2 cells (21). To determine whether ICP0 can activate the HIV-1 LTR by cooperation with other transactivating factors, especially the NF-κB binding proteins, we have transfected the p23tkCAT plasmid into A3.01 cells (Fig. 5B) and examined its expression upon transactivation with different inducers. PMA treatment of the transfected cells, which activates NF-κB proteins, resulted in a 3-fold activation of the CAT activity, whereas cotransfection with ICP0 (under the control of a cytomegalovirus promoter) increased the CAT activity by 6.5-fold. The combined induction with PMA and ICP0 resulted in an 11-fold increase of the CAT activity. The synergism between ICP0 and NF-κB binding proteins was even more apparent when the HIV-1 LTR CAT plasmid was used. While both PMA and ICP0 stimulate the CAT activity about 3- to 4-fold, combination of the two results in 14-fold induction of the HIV-1 LTR promoter (Fig. 5B). The cotransfection of ICP0 with plasmid pBLCAT2, which contains the minimal tk promoter and which is expressed only at very low levels in HSV-1-infected cells, enhanced the levels of CAT activity only 2-fold, and a combination of PMA and ICP0 increased the expression about 4-fold. These results show that ICP0, which is a rather promiscuous transcriptional transactivator (for a review, see reference 6), acts in cooperation with other transcriptional factors bound to the HIV-1 promoter, such as PMA-induced NF-κB binding proteins. When similar experiments were performed with the ICP4 expression plasmid, no significant

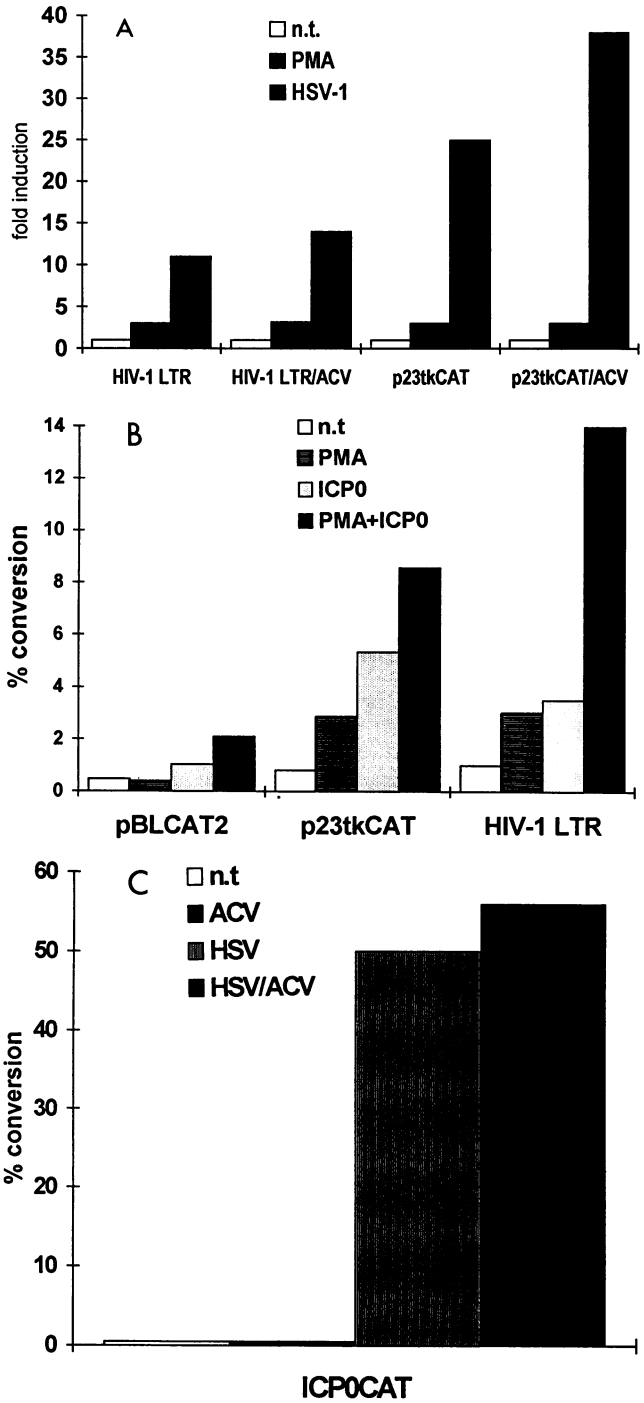

FIG. 5. (A) Effect of ACV on the HSV-1-mediated activation of the p23tkCAT and the HIV-1 LTR CAT plasmid. Cells (2×10^7) were transfected with 10 μg of the reporter plasmid, and 24 h after transfection they were treated with PMA (50 ng/ml for 16 h) or infected with HSV-1 (MOI, 3) or left untreated (n.t.). The same experiment was performed in the presence of ACV (30 μM), which was added to all samples 24 h after transfection. The graph shows fold induction over the value of the untreated samples. The basal CAT activities of the uninduced reporter plasmids (using 50 μg of cell lysates at 37°C for 2 h), expressed in counts per minute of acetylated [^{14}C]chloramphenicol, were as follows: HIV LTR CAT, 3,100; HIV LTR CAT/ACV, 3,500; p23tkCAT, 1,020; and p23TKCAT/ACV, 960. The results represent the average of at least three transfection experiments. (B) Synergism between the NF-κB and ICP0. A3.01 cells (1×10^7) were transfected with 3 μg of the reporter plasmid and 6 μg of either the nonspecific DNA or pGH94 (*ICP0* gene under control of the cytomegalovirus promoter). Where stated, 24 h after transfection cells were treated with PMA (50 ng/ml). Values on the *y* axis show the percent conversion of the [^{14}C]chloramphenicol to the acetylated form with 50 μg of cell lysate at 37°C for 2 h. (C) ACV treatment does not affect the activity of the ICP0 promoter in HSV-1-infected cells. A3.01 cells (2×10^7) were transfected with 12 μg of the ICP0-CAT reporter plasmid, and after 24 h (18) cells were split into four portions; the first was left untreated (n.t.), the second was treated with 30 μM ACV, the third was infected with HSV-1 (3 MOI), and the fourth was infected with HSV-1 in the presence of 30 μM ACV. CAT activity was measured with 10 μg of the cell lysate at 37°C for 30 min. Values represent averages of three transfections.

increase in CAT activity was observed either with ICP4 alone or with ICP4 in combination with PMA, indicating that ICP4 alone is not able to activate the p23tkCAT or the HIV-1 LTR CAT plasmids in A3.01 cells (21). These results are in agreement with the finding that the HIV-1 LTR promoter does not contain the ICP4 DNA binding motif (7).

The cotransfection studies have shown that ICP0, but not ICP4, can transactivate expression of the HIV-1 LTR promoter region (11–13), and we have shown in this report that the presence of the NF-κB enhancer region either alone or in combination with the LBP-1 region is sufficient for the ICP0-mediated transactivation. However, using the ICP4 deletion mutant of HSV-1, Albrecht et al. (2) have shown that the function of ICP4, and not that of ICP0, is essential for the augmentation of HIV-1 replication in T cells. The reason for this discrepancy is not obvious; it may, however, indicate either that the effect of the IE genes in the context of viral infection and the effect of the isolated transfected genes are not identical or that the expression of an early and late gene(s), which is blocked in the ICP4-defective HSV-1 mutant, is required for the HIV-1 induction.

In the course of this study, we have tested two HSV-1 mutants for their ability to activate HIV-1 replication in ACH-2 cells. The first was the VP16 mutant, *in*1814 (1). Since the virion protein VP16 is one of the most efficient transactivators, we wanted to determine whether this gene also participates in the activation of the HIV-1 LTR. However, the VP16 mutant was able to induce HIV-1 expression nearly as efficiently as the parental HSV-1 (21). These results, together with the finding that VP16 overexpression did not activate HIV-1 LTR in a transient-transfection assay (data not shown), exclude the participation of VP16 in the activation of the HIV-1 provirus. The second mutated virus tested was the protein kinase mutant of HSV-1 (17). Since the induction of NF-κB binding activity is at least partially a consequence of phosphorylation of IκB (8), it was of interest to determine whether the virus-encoded kinase plays a role in the activation of NF-κB binding and the HIV-1 induction process. However, both the kinase mutants and the parental virus were able to induce expression of the HIV-1 provirus and NF-κB binding proteins to a similar extent (21).

The results of this study show that, while the inhibition of expression of late genes does not affect the HSV-1-mediated induction of HIV-1 provirus, the levels of NF-κB and HLP-1 proteins are significantly reduced when the later stages of HSV-1 infection and virus production are inhibited. We believe that these results are not an indication that the NF-κB and HLP-1 proteins do not play any role in the HSV-1-mediated activation of HIV-1 provirus but rather

support our previous observation that even very low levels of NF-κB proteins can efficiently activate the expression of HIV-1 provirus (19). Furthermore, we suggest that the activation of HIV-1 provirus by HSV-1 is mediated by a combined effect of several transactivators, such as NF-κB, HLP-1, and ICP0 proteins. The complementation and cooperation between the cellular transactivators such as NF-κB binding proteins and the virus-encoded IE gene, i.e., ICP0, allow effective HIV-1 transactivation even under conditions in which the HSV-1 replication is impaired and the levels of NF-κB and HLP-1 transactivators are significantly reduced. The observation that ACV-mediated inhibition of HSV-1 replication does not affect the stimulation of HIV-1 provirus expression but, in contrast, enhances the production of HIV-1 virions is of particular interest, since a number of clinical protocols used in AIDS therapy combine ACV and antiretrovirus treatment.

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