The Herpes Simplex Virus Immediate-Early Protein, ICP4, Is Required To Potentiate Replication of Human Immunodeficiency Virus in CD4⁺ Lymphocytes

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The interaction of human immunodeficiency virus (HIV) and herpes simplex virus (HSV) was investigated in an acute whole-virus coinfection system. CD4⁺ lymphoid CEM cells were infected with HIV-1 and, 24 h later, superinfected with HSV-1 (strain KOS) or HSV mutants possessing defined deletions in genes specifying the immediate-early transcriptional regulatory proteins ICP0, ICP4, or ICP27. Marked potentiation of HIV replication was demonstrated with the KOS strain, the ICP0 mutant, and the ICP27 mutant, but not with the ICP4 mutant, indicating that ICP4 is essential and ICP0 and ICP27 are nonessential for this effect. These studies demonstrate that HSV can be a potent stimulator of HIV replication and gene expression in coinfecting CD4⁺ cells through the activity of the HSV regulatory protein ICP4.

Much attention has been focused on the possibility that cofactors may be important in the pathogenesis of acquired immunodeficiency syndrome (AIDS) (1, 13, 35, 40, 41, 44). Among potential cofactors, herpesviruses are often mentioned because they are ubiquitous, have a particularly high prevalence in certain high-risk populations (15, 21, 32, 33), cause increased morbidity and mortality in patients with AIDS (2, 17, 42), and moreover, are known to specify at least three potent transcriptional transactivators (9, 19, 31). Further, cytomegalovirus, varicella-zoster virus, Epstein-Barr virus, and herpes simplex virus (HSV), among other DNA viruses, have been shown to be able to transactivate the human immunodeficiency virus (HIV) long terminal repeat (LTR) in transfection assays with the LTR linked to the chloramphenicol acetyltransferase (*cat*) indicator gene (4, 11, 16, 22, 23, 25, 28).

HSV has received the most experimental scrutiny in this regard, with reports that both an intact HSV-1 genome and certain immediate-early (5, 6, 38) transregulatory proteins (ICP0 and ICP4) can transactivate the HIV LTR in transfection assays in nonlymphoid cells (22, 23, 28). Although the results of transfection assays indicate what effects specific HSV proteins may have on expression from the HIV LTR, these tests do not necessarily reflect the activities of the same proteins in the context of the HSV genome, i.e., during coinfection with both HIV and HSV.

We have been interested in HIV-HSV interactions in one of the natural targets of HIV, the CD4⁺ lymphoid cell, and in investigating intact virus-virus interactions in such systems. Previously, we reported that HIV superinfection of a CEM lymphoid line persistently infected with HSV resulted in significant potentiation of HIV replication (M. A. Albrecht, J. M. Gillis, N. T. Andrea, and S. M. Hammer, 27th Intersci. Conf. Antimicrob. Agents Chemother, abstr. no. 839, 1987). In the current study, we describe an acute coinfection system in which the interactions of HIV with HSV type 1 (HSV-1) or selected isogenic HSV-1 deletion mutants were examined in an effort to determine which of the three

recognized HSV transregulatory proteins (ICP0, ICP4, or ICP27) (5, 6, 19, 37, 38) play a role in the potentiation of HIV.

MATERIALS AND METHODS

Cells. CEM cells (CD4⁺ lymphoid cells) were maintained in RPMI 1640 medium supplemented with penicillin, streptomycin, L-glutamine, and 10% fetal calf serum (FCS) and were mycoplasma-free.

Viruses. Virus strains included HIV-1 (HTLV-III_B) (courtesy of R. C. Gallo, Bethesda, Md.) propagated in H9 cells and HSV strains passaged in Vero cells with the following titers: wild-type HSV-1 strain (KOS), 8.2 log₁₀ PFU/ml; the ICP0 deletion mutation *dlx3.1* (Δ ICP0), 8.5 log₁₀ PFU/ml; the ICP4 deletion mutant *d120* (Δ ICP4), 8.2 log₁₀ PFU/ml; and the ICP27 deletion mutant *5dl1.2* (Δ ICP27), 8.2 log₁₀ PFU/ml.

Establishing acute coinfection. (i) HIV infection of CEM cells. On day 0, 20 × 10⁶ CEM cells were incubated with 2 ml of filtered cell-free supernatant harvested from an HIV-infected H9 cell culture for 1 h at 37°C (reverse-transcriptase [RT] activity of inoculum, 5.8 to 6.0 log₁₀ cpm/ml). Control CEM cells were mock infected with cell-free supernatant from an uninfected H9 cell culture. The cells were then washed with medium not supplemented with FCS and suspended to a concentration of 5 × 10⁵/ml.

(ii) HSV superinfection of CEM cells. Twenty-four hours after HIV infection, CEM cells were superinfected with the wild-type strain of HSV-1 (KOS) or with mutant *dlx3.1*, *d120*, or *5dl1.2* containing deletions in the genes specifying ICP0, ICP4, and ICP27, respectively, at a multiplicity of infection of 2.5 PFU/cell. A total of 10 × 10⁶ CEM cells were incubated with 1 ml of HSV strain for 1 h at 37°C, washed, and suspended to a concentration of 5 × 10⁵/ml. Control cultures included cells exposed to HSV alone and uninfected CEM cells. Cultures were passed every 3 to 4 days.

RT activity. RT activity was measured as described by Poiesz et al. (29). Virus was precipitated from culture supernatants with 4 M NaCl-30% polyethylene glycol and placed on ice overnight. Following centrifugation and resus-

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pension, a solution of 0.9% Triton X-100–1.5 M KCl was added to disrupt virions. A portion of the disrupted virus solution was added to a solution containing 50 μ g of template primer poly(dA · dT) or 50 μ g of poly(A · dT) per ml. [3 H]dTTP (16 Ci/mmol; New England Nuclear, Boston, Mass.) was introduced to the reaction mixture, and counts per minute were determined in a Beckman model LS680 liquid scintillation counter.

HIV Ags in supernatant. HIV antigens (Ags) in culture supernatant fluids were detected by using the Abbott HIV Ag sandwich solid-phase enzyme immunoassay. Polystyrene beads coated with human antibody (Ab) to HIV were incubated with 200 μ l of culture supernatant or nonimmune serum at room temperature for 16 to 20 h. Following incubation, unbound materials were aspirated and beads were washed. Rabbit anti-HIV Ab (200 μ l) was then added to each well and incubated for 4 h at 40°C, after which unbound materials were aspirated and the beads were washed. The third incubation entailed dispensing 200 μ l of goat anti-rabbit immunoglobulin G complexed with horseradish peroxidase into the wells for 2 h at 40°C. Beads were subsequently transferred to assay tubes, and 300 μ l of *O*-phenylenediamine solution was added and incubated at room temperature for 30 min. The enzyme reaction was terminated with the addition of 1 ml of 1 N sulfuric acid to each tube. The intensity of the color reaction was read by using a spectrophotometer at 492 nm. Samples with absorbance values equal to or greater than the calculated cutoff value were considered reactive for HIV Ags, and protein values, derived from the absorbance results, were determined for each sample.

Immunofluorescence for cell-associated HIV p24. HIV p24 Ag and HSV Ag expression were assessed by indirect immunofluorescence. Infected and control cells were washed in phosphate-buffered saline, air-dried on glass slides, and fixed in 50% methanol–50% acetone. Slides were incubated with a murine monoclonal anti-HIV p24 Ag (Du Pont, Billerica, Mass.) or a human polyvalent anti-HSV serum for 30 min at 30°C in a humidified atmosphere. They were stained with a fluorescein isothiocyanate-conjugated F(ab)₂ goat anti-mouse or anti-human immunoglobulin, respectively, counterstained with Evans blue, and examined under an Olympus BH2 fluorescent microscope. The percent of viral Ag-positive cells were determined by counting a minimum of 100 cells per test.

HSV titer. Samples (0.5 ml) from extracts of total cell cultures were obtained on days 1 through 10. After freeze-thawing of samples three times to release intracellular virus, serial 10-fold dilutions of test samples in cell culture medium were placed in duplicate 0.1-ml amounts onto Vero cell monolayers in 35-mm, six-well plates (Costar, Cambridge, Mass.). Following a 1-h adsorption at 37°C, monolayers were overlaid with 0.5% agarose in Eagle minimal essential medium with 5% FCS. Plates were incubated for 4 days at 37°C, stained with neutral red, and reincubated. Plaques were counted on day 5.

Cell viability. Daily cell viability counts were determined in each culture from days 0 to 10 and subsequently every 3 to 4 days by the trypan blue dye exclusion technique.

RESULTS

KOS superinfection of HIV-infected CEM cells. The KOS wild-type strain of HSV was used in the first series of experiments. Compared with CEM cells exposed to HIV alone (CEM/HIV), the coinfection culture (CEM/HIV/KOS)

demonstrated a marked enhancement of HIV replication. By day 4, the coinfecting culture exhibited an RT activity of 3.5 log₁₀ cpm/ml, compared with undetectable RT activity in the singly infected culture (Fig. 1A). This trend of accelerated HIV replication continued through day 7, when peak RT activity (5.6 log₁₀ cpm/ml) was achieved in the coinfecting culture. Superinfection with HSV resulted in a 4-day acceleration in the time of induction and a 3-day acceleration in the time to peak RT activity. In the singly infected culture, 7 days were required before RT activity (3.2 log₁₀ cpm/ml) was demonstrable. The supernatant HIV Ag assay confirmed the acceleration and higher level of HIV replication in the coinfecting culture (CEM/HIV/KOS) in the 10-day test period (Fig. 1B). On day 2, the coinfecting culture supernatant first expressed measurable HIV Ag, and by day 4, this culture had an Ag level 10,000 times greater than that of the culture infected with HIV alone. On day 7, when RT was first detectable in the singly infected culture, the supernatant HIV Ag level was measured at 1,900 pg/ml (Fig. 1A and B), which represented a lag of 4 days compared with the coinfecting culture. Cellular HIV p24 Ag expression followed the same accelerated pattern of expression observed for both RT and supernatant HIV Ag (Fig. 1C). By day 5, 18% of the cells in the coinfecting culture expressed HIV p24 Ag, compared with 1.4% for the singly infected culture, a 13-fold difference ($P < 0.01$) (Student's *t* test). Although day 10 was the time at which the highest frequency (33%) of HIV p24-positive cells was observed in the CEM/HIV cultures, prior to day 9 fewer than 10% of the cells were p24 Ag positive. Notably, the slopes of the induction curves were similar in CEM/HIV/KOS cells for all three activities tested, indicating that HSV potentiates induction of HIV and has little effect on the rate of HIV replication once induced. The coinfecting culture developed multinucleated giant cells and prominent ballooning of cell membranes by day 3, which signaled the cytopathic effects (CPEs) characteristic of HIV infection. These changes were ascribed to HIV because they were not seen in the cultures singly infected with HSV but were seen (albeit after a delay) in cultures infected only with HIV. The possibility of additive CPE in cultures coinfecting with HIV and HSV could not be excluded, however. The HIV-induced CPE preceded any decrease in cell viability, which was delayed until day 6, whereas the culture singly infected with HIV resembled uninfected control cells and maintained $\geq 80\%$ viability (Fig. 1D).

We then examined HSV mutants with deletions in the immediate-early genes specifying ICP0, ICP4, and ICP27 to determine which of these proteins were required for enhancement of HIV expression. Deletion mutants were used because unlike temperature-sensitive mutants, they exhibit no "leak" at temperatures optimal for cellular growth (20). Wild-type strain KOS was always included as a positive control in these tests.

Δ ICP0 superinfection of CEM/HIV cells. The ICP0 deletion mutant *dI*x3.1 (Δ ICP0) was compared with KOS for its ability to potentiate HIV replication. This mutant fails to induce the synthesis of ICP0 because the transcriptional start site and much of the ICP0 coding sequence are deleted in both copies of the gene (38). Since ICP0 is not essential for viral growth, however, *dI*x3.1 (Δ ICP0) is replication competent (38). Accelerated HIV replication as shown by RT activity was observed in both the CEM/HIV/ Δ ICP0 and the CEM/HIV/KOS coinfecting cultures (Fig. 1A). The Δ ICP0-superinfected culture (CEM/HIV/ICP0) had a more rapid induction of RT activity (day 3) than the CEM/HIV/KOS culture, which suggested a trend for even greater enhance-

FIG. 1. HIV RT activity (A), supernatant HIV Ag (B), cellular expression of HIV p24 Ag (C), and cell viability (D) in CEM cells infected with HIV alone or coinfecting with HIV and KOS or Δ ICP0. Values shown represent the mean of three experiments. IFA, Immunofluorescence.

ment of HIV replication provided by Δ ICP0. On day 5, both the CEM/HIV/ Δ ICP0 and the CEM/HIV/KOS coinfecting cultures had equivalent levels of RT activity ($4.5 \log_{10}$ cpm/ml) (Fig. 1A), whereas 7 days were required for the CEM/HIV culture to exhibit demonstrable RT activity. Moreover, this level remained substantially lower than that of the coinfecting cultures.

HIV Ag levels in culture supernatants confirmed the results of RT assays (Fig. 1B). By day 4, CEM/HIV/KOS and CEM/HIV/ Δ ICP0 cultures demonstrated an HIV Ag level of 10,000 and 14,000 pg/ml, respectively, compared with 400 pg/ml in the CEM/HIV culture. Cellular HIV p24 Ag expression further confirmed the augmentation of HIV expression by Δ ICP0 (Fig. 1C). The curves for the two superinfected cultures were almost superimposable. On day 5, the percentage of cells expressing p24 HIV Ag in CEM/HIV/ Δ ICP0, CEM/HIV/KOS, and CEM/HIV cultures was 23, 18, and 1.4%, respectively. By contrast, cellular HIV p24 Ag was seen in increased numbers of CEM/HIV cells (5%) only after 6 days.

Both coinfecting cultures developed characteristic HIV-induced CPE by day 3. This marked CPE preceded any decrease in cell viability, which was delayed until day 6 when CEM/HIV/ Δ ICP0 and CEM/HIV/KOS cultures had 82 and 81% cell viability, respectively, compared with 94% viability and minimal CPE for CEM/HIV (Fig. 1D). By day 10, both coinfecting cultures had a cell viability of <5%, whereas the HIV-infected (CEM/HIV) culture maintained a viability of 89% ($P < 0.01$ Student's *t* test). CEM cells exposed to KOS or Δ ICP0 alone, in contrast to the coinfecting cultures, did not exhibit characteristic CPE and underwent a substantially slower decrease in cell viability

which paralleled the degree of HSV-induced lysis resulting from productive infection.

The above findings demonstrated that the ICP0 deletion mutant (Δ ICP0) was as efficient as KOS in potentiating HIV replication, as reflected by the equivalent RT and supernatant and cellular HIV Ag levels (Fig. 1A, B, and C), indicating that ICP0 is not essential for the potentiation of HIV replication by HSV.

Δ ICP4 superinfection of CEM/HIV cells. We next attempted to determine whether ICP4 was essential for potentiation by utilizing a deletion mutant, *d120* (Δ ICP4), lacking both copies of the ICP4 gene. Since ICP4 is essential for virus growth, *d120* (Δ ICP4), in contrast to *dlx3.1* (Δ ICP0), is replication incompetent. Phenotypic analysis of *d120* (Δ ICP4) has shown that this mutant is blocked in gene expression at a point prior to HSV early gene expression and viral DNA synthesis. For this reason, only a fixed number of DNA templates (the input number) was introduced into cells upon superinfection with Δ ICP4.

The ICP4 deletion mutant failed to augment HIV expression, as shown by the results of RT assays (Fig. 2A), which demonstrated that the culture coinfecting with HIV and Δ ICP4 (CEM/HIV/ Δ ICP4) had a level of RT activity comparable to that of the culture infected with HIV alone. In contrast, KOS superinfection again resulted in accelerated expression of HIV RT activity (Fig. 2A). Supernatant HIV Ag levels are shown in Fig. 2B. Both the CEM/HIV/ Δ ICP4 culture and the CEM/HIV culture demonstrated delayed Ag expression compared with the CEM/HIV/KOS culture (400, 400, and 10,000 pg/ml, respectively, on day 4). Induction of supernatant HIV Ag expression in cultures superinfected with KOS was first evident on day 3, whereas no antigen

FIG. 2. HIV RT activity (A), supernatant HIV Ag (B), cellular expression of HIV p24 Ag (C), and cell viability (D) in CEM cells infected with HIV alone or coinfecting with HIV and KOS or Δ ICP4. Values shown represent the mean of three experiments. IFA, Immunofluorescence.

increase was detected until day 6 in CEM/HIV/ Δ ICP4 or CEM/HIV cultures. Moreover, peak levels were reached by day 7 in CEM/HIV/KOS cultures, but comparable levels were not detected until day 10 or later for CEM/HIV/ Δ ICP4 and CEM/HIV cultures. Cellular expression of HIV p24 Ag confirmed the lack of potentiation of HIV replication by Δ ICP4 (Fig. 2C). The CEM/HIV/KOS, CEM/HIV/ Δ ICP4, and CEM/HIV cultures demonstrated 45, 10, and 7% of cells expressing p24 HIV Ag on day 6, respectively. The number of Ag-positive cells in the CEM/HIV/ Δ ICP4 culture paralleled that found in the CEM/HIV culture throughout the 10-day monitoring period.

The CEM/HIV/ Δ ICP4 coinfecting culture exhibited minimal CPE by day 5 and maintained 87 to 91% cell viability throughout the 10-day test, which paralleled the results for the CEM/HIV culture (Fig. 2D).

These results suggest that ICP4 is essential for potentiation of HIV replication and Ag expression in CEM lymphoid cells. As mentioned above, however, unlike the ICP0 mutant (Δ ICP0), the ICP4 mutant is replication incompetent since ICP4 is required for productive infection. One could argue, therefore, that the inability of Δ ICP4 to accelerate HIV gene expression is a consequence of the failure of the mutant to replicate and not of the absence of ICP4.

Δ ICP27 superinfection of CEM/HIV cells. Consequently, a subsequent experiment was conducted in which a mutant carrying a deletion in ICP27, 5d/1.2 (Δ ICP27), was used. ICP27 is the third HSV immediate-early protein known to be involved in the regulation of viral gene expression. The ICP27 deletion mutant 5d/1.2 is replication incompetent, since ICP27 is an essential replicative protein, but has fully intact genes for ICP0 and ICP4 (34, 37). The results of this

experiment, which included repeat tests of the Δ ICP0 and Δ ICP4 mutants, are shown in Fig. 3. CEM/HIV/KOS, CEM/HIV/ Δ ICP0, and the CEM/HIV/ Δ ICP27 cultures demonstrated clear potentiation of HIV infection, with earlier positive RT activities than the CEM/HIV and the CEM/HIV/ Δ ICP4 cultures, which were first RT positive on day 9. A minimum 4-day lag was evident between the potentiation of RT activity in the KOS, Δ ICP0, and Δ ICP27 superinfected cultures and the CEM/HIV and CEM/HIV/ Δ ICP4 cultures (Fig. 3A). The CEM/HIV/ Δ ICP0 coinfecting culture again exhibited the earliest positive RT activity (Fig. 3B; see also Fig. 1B). HIV supernatant Ag levels detected in the CEM/HIV/KOS, CEM/HIV/ Δ ICP0, and CEM/HIV/ Δ ICP27 cultures showed a dramatic increase between days 3 and 4 and were, after day 4, virtually equivalent throughout the remainder of the experiment (Fig. 3B). Furthermore, the CEM/HIV/ Δ ICP27 culture had a higher HIV Ag level (60,000 pg/ml) on day 4 than the CEM/HIV/KOS and CEM/HIV/ Δ ICP0 cultures (9,900 and 14,800 pg/ml, respectively). In addition, the CEM/HIV/ Δ ICP4 and CEM/HIV cultures expressed an HIV Ag level of only 400 pg/ml on day 4, which represented a 150-fold difference from that of CEM/HIV/ Δ ICP27 (60,000 pg/ml). It was therefore evident that the replication-incompetent ICP27 mutant was as efficient an enhancer of HIV expression as the wild-type virus or Δ ICP0. The cellular immunofluorescence results for HIV p24 Ag (Fig. 3C) confirmed the substantial acceleration of HIV infection within CEM/HIV/ Δ ICP27, CEM/HIV/ Δ ICP0, and CEM/HIV/KOS cultures. On day 6, 54, 44, and 45% of cells were positive for HIV p24 Ag, respectively, compared with less than 5% for CEM/HIV/ Δ ICP4 and CEM/HIV cultures. Based on these and previous results, it appeared likely that

FIG. 3. HIV RT activity (A), supernatant HIV Ag (B), cellular expression of HIV p24 Ag (C), and cell viability (D) in CEM cells infected with HIV alone or coinfecting with KOS, Δ ICP0, Δ ICP4, and Δ ICP27. Values shown represent the results of a representative experiment in which KOS and all mutant HSV strains were examined concurrently. IFA, Immunofluorescence.

the presence of a functional ICP4 gene within the Δ ICP27 mutant accounted for the potentiation of HIV.

Cell viability in those coinfecting cultures which had demonstrated potentiation of HIV replication (CEM/HIV/ Δ ICP27, CEM/HIV/ Δ ICP0, and CEM/HIV/KOS) underwent a progressive decrease, noted initially on day 6 and reaching less than 10% by day 10 (Fig. 3D). In contrast, the CEM/HIV/ Δ ICP4 and CEM/HIV cultures, in which no potentiation of HIV expression was demonstrated, maintained greater than 90% cell viability throughout the 10-day test (Fig. 3D).

In order to rule out the possibility that the differences between cultures superinfected with Δ ICP4 and Δ ICP27 were not a consequence of unanticipated growth of the replication-incompetent ICP27 deletion mutant, culture fluids were assayed in ICP27-expressing 3-3 cells, which complement the defect in the ICP27 deletion mutant (19). No infectious virus was detected, demonstrating that replication of the Δ ICP27 mutant was not the cause of the acceleration of HIV. Rather, the presence of ICP4 in the Δ ICP27 genome produced the acceleration.

In order to assess the growth abilities of the replication-competent herpesviruses KOS and Δ ICP0 in these studies, total infectious virus in CEM/HIV/KOS, CEM/HIV/ Δ ICP0, CEM/KOS, and CEM/ Δ ICP0 cultures were determined by standard plaque assay. Expression of HSV-specific antigen was assessed in both the replication-competent (KOS and Δ ICP0) and -incompetent (Δ ICP4 and Δ ICP27) viruses (Table 1). Comparable HSV titers were demonstrated in the coinfecting cultures (CEM/HIV/KOS and CEM/HIV/ Δ ICP0) and in CEM cultures infected with HSV or Δ ICP0 alone. Similarly, HSV Ag-positive cells were present in approxi-

mately equal percentages in HSV-superinfected cultures and in CEM cells exposed to HSV alone. Thus, neither the level of HSV replication nor HSV Ag expression was affected by coinfection with HIV. The stable HSV titers and the relatively low percentage of HSV Ag-positive cells seen are consistent with previous reports of the limited permissiveness of CEM cells for HSV compared with Vero cells and demonstrated, furthermore, the ability of HSV to establish stable, persistent infections in CEM cells with minimal, if any, reduction in cell viability (14).

DISCUSSION

HSV superinfection enhances HIV replication. These results demonstrate that HSV (KOS) and deletion mutants of HSV lacking ICP0 and ICP27 are able to potentiate HIV

TABLE 1. HSV replication results

Culture	HSV titer ($\log_{10}$ PFU/ml)		% HSV Ag-positive cells	
	Day 3	Day 7	Day 3	Day 7
CEM/KOS	5.6	5.2	17	22
CEM/HIV/KOS	5.5	5.5	15	27
CEM/ Δ ICP0	4.9	5.1	11	11
CEM/HIV/ Δ ICP0	4.8	6.6	12	8
CEM/ Δ ICP4	NT ^a	NT	2	6
CEM/HIV/ Δ ICP4	NT	NT	1	9
CEM/ Δ ICP27	NT	NT	18	17
CEM/HIV/ Δ ICP27	NT	NT	15	34

^a NT, Not tested; ICP4 and ICP27 are not replication competent.

infection markedly within coinfecting lymphoid cells. In contrast, the replication-incompetent ICP4 deletion mutant (*d120*) had no stimulatory effect on HIV expression. The failure of this mutant to augment HIV expression suggests that either ICP4 or virus replication is necessary for this enhancement. It was concluded that functional ICP4 and not virus replication must be essential for HIV potentiation, since the ICP27 mutant (*5d1.2*), which is replication incompetent but possesses functional ICP0 and ICP4 genes, was an especially strong inducer of HIV expression. Thus, the limited permissiveness of CEM cells for fully replicative HSV is not an important determinant for the potentiation of HIV in our system, which in fact is essentially dependent on ICP4 gene expression. Although ICP0 is not necessary for enhancing HIV replication, the expression of ICP0 and ICP4 by the ICP27 deletion mutant may have an additive or synergistic effect on HIV expression. Alternatively, the ICP27 mutant may lack an inhibitory function which may result in even more efficient potentiation of HIV replication than either KOS or the ICP0 deletion mutant. These studies also indicate that the HSV virion component is not sufficient for potentiation of HIV growth. ICP4 deletion mutant virions (6) contain this component, VP16, which upregulates immediate-early genes together with cellular elements through *cis*-acting elements (TAATGARAT) in immediate-early promoters (7). HIV contains an NF- κ B element in its LTR (24), which may be similar to the TAATGARAT elements in immediate-early promoters (27).

Role of immediate-early HSV genes in HSV transactivation. Of the five immediate-early proteins of HSV (ICP0, ICP4, ICP22, ICP27, and ICP47), only ICP0, ICP4, and ICP27 have been shown to function in the regulation of HSV gene expression. Together, these three proteins have been implicated in the activation and repression of all three major classes of HSV genes (immediate-early, early, and late). In transient expression assays, ICP0 has been shown to be a potent transactivator of all three classes of viral genes (9, 18, 31) and to act synergistically with ICP4 in the activation of a variety of HSV promoters (7, 10, 26). Despite its strength as a transactivator, however, deletion mutants which lack ICP0, such as *d1x3.1* (Δ ICP0), used in these studies, are replication competent and exhibit no clear phenotypic alterations compared with the wild-type virus at high multiplicities (38). They are growth impaired at low multiplicities, however, and induce the synthesis of lower levels of selected early proteins, viral DNA, and most late proteins than does wild-type virus (43; W. Sacks and P. Schaffer, unpublished observations). Although ICP0 is not essential for productive infection, it has been implicated in the reactivation of HSV from latent infection (3, 12; D. Leib, D. Coen, C. Bogard, K. Hicks, D. Yoeger, D. Knipe, K. Tyler, and P. A. Schaffer, J. Virol., in press).

Temperature-sensitive (*ts*) and deletion ICP4 mutants are not viable under nonpermissive conditions, demonstrating an essential role for this protein in HSV replication (5, 8, 30). Specifically, ICP4 mutants fail to induce the synthesis of early and late proteins and viral DNA and to down regulate immediate-early protein synthesis (5, 8, 30). Transient expression assays have confirmed the ability of ICP4 to transactivate early and late genes (7, 9, 10, 26). ICP4-mediated regulation of viral gene expression occurs at the level of transcription and may involve the binding of ICP4—either directly or indirectly—to specific target sequences on the viral genome (6, 20).

Like *ts* ICP4 mutants, *ts* ICP27 mutants are not viable at the nonpermissive temperature, demonstrating an essential

role for this protein in productive infection (37). Although early studies of the role of ICP27 in viral gene expression by using transient expression assays revealed no significant *trans*-regulating activity for protein (5, 9), more recent studies indicate that ICP27 can both enhance (10) and repress (5, 9, 34, 39) expression of viral genes driven by ICP0 and ICP4. Thus, the results of studies with *ts* mutants and transient expression assays implicate ICP27 as well as ICPs 0 and 4 in the regulation of gene expression in the HSV system.

HSV-induced transactivation of the HIV LTR in transient expression assays. The transactivation of the HIV LTR by herpesviruses in nonlymphoid cells was first described by Gendelman et al. (11) in transient expression assays with a cloned HSV DNA fragment containing the genes for ICP0, ICP4, and ICP27 and a cloned VZV DNA fragment containing a gene encoding the VZV equivalent of the HSV ICP4 gene. Subsequently, Mosca et al. demonstrated that HSV-1 is capable of transactivating a chimeric HIV LTR-*cat* reporter gene both in transient expression assays and when the gene is stably introduced into Vero and Ltk⁻ cells (22). Efforts by Mosca et al. to determine which HSV-1 gene products were involved in HIV LTR induction led to the conclusion that ICP0 alone or ICP0 in combination with ICP4—but not ICP4 alone—was able to induce the LTR. These findings are in sharp contrast to our findings in coinfection studies with mutants lacking ICP0 and ICP4, in which ICP4 but not ICP0 was found to be essential for transinduction. In more recent transient expression studies, Ostrove et al. (28) demonstrated that both an intact HSV-1 genome and the transfected ICP4 gene were able to induce transactivation of the HIV LTR-*cat* system within SW480 cells in the absence of the HIV Tat protein, whereas ICP0 had less stimulatory effect than ICP4 and ICP27 provided no augmentation. Furthermore, HSV and both the cloned ICP0 and ICP4 genes but not the ICP27 gene enhanced HIV replication in HeLa cells transfected with an infectious clone of HIV. These results were variable in different cell lines but indicated that ICP0 and ICP4 but not ICP27 were capable of transactivation of the HIV LTR (28).

ICP4 is essential for HIV potentiation in lymphoid cells. Our results with coinfecting CEM cells support Ostrove's plasmid transfection findings in the following manner: (i) HSV (KOS) is capable of augmenting HIV replication; (ii) ICP4 is necessary for HIV potentiation, (iii) ICP27 is not essential for induction; and (iv) fully productive HSV infection is not required for the potentiation of HIV replication. The fundamental difference between our results and the work of Mosca et al. and Ostrove et al. is our finding that the ICP0 gene is not required to stimulate HIV replication.

Another study designed to elucidate the mechanism by which HSV transinduces the HIV LTR is that of Nabel et al. (25). As in previous studies, these investigators found that the combination of ICP0 and ICP4 but not ICP4 alone was able to transduce an HIV LTR-*cat* construct in transient expression assays. Efforts to identify potential *cis*-acting target sequences responsive to ICP0 in the HIV LTR were unsuccessful, leading these investigators to conclude that ICP0 does not function through a single, defined, *cis*-acting element. The identity of the specific *cis*-acting signals within this sequence that respond to HSV infection in general and ICP4 specifically remain to be determined.

The results of our coinfection studies are totally consistent with the demonstrated activities of ICP0, ICP4, and ICP27 in the regulation of HSV gene expression. As noted above, only ICP0 and ICP4 exhibit transinducing activity. ICP27

appears to modulate or fine-tune the activities of ICP0 and ICP4 but exhibits no universal transactivating activity. Notably, studies of ICP4 deletion mutants have demonstrated that ICP0 exhibits no transinducing activity in the absence of ICP4 in the context of the mutant genome (6). Why ICP0 alone exhibits this activity in transient assays but not in the genome of an ICP4 deletion mutant is not known. Additionally, the observation that potentiation of HIV replication by ICP0 and ICP4 (in the genome of the ICP27 deletion mutant) was greater than that by ICP0 or ICP4 alone is consistent with the fact that these proteins function synergistically to stimulate HSV gene expression.

Although it is known that ICP4 transactivates HSV gene expression at the transcriptional level (6), the mechanism by which ICP4 potentiates HIV replication is unclear. Transactivation of the HIV LTR is the likely mechanism, but whether this is a result of an HSV gene product binding directly to *cis*-acting elements in the HIV LTR or an event mediated indirectly through cellular transcription factors needs to be defined further (24, 25). Finally, posttranscriptional events affecting HIV expression (25, 28, 36) may be modified or otherwise influenced by HSV gene products to affect HIV replication.

Although the clinical importance of HIV-HSV interactions in HIV-related disorders remains to be determined, should these interactions prove clinically relevant, these studies provide a target protein, ICP4, for efforts to block the potentiation of HIV by HSV.

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LITERATURE CITED

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