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## Transactivation of the Human Immunodeficiency Virus Promoter by Human Herpesvirus 6 (HHV-6) Strains GS and Z-29 in Primary Human T Lymphocytes and Identification of Transactivating HHV-6(GS) Gene Fragments

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**Human herpesvirus 6 (HHV-6) can activate the human immunodeficiency virus (HIV) promoter and accelerate cytopathic effects in HIV-infected human T cells. This study examines the regions of the HIV promoter required for HHV-6 transactivation in a heterogeneous population of primary human T lymphocytes with or without antigenic stimulation. Two different strains of HHV-6, GS and Z29, transactivated the HIV promoter. The GS strain transactivated the promoter in both stimulated and resting T cells, while the Z29 strain increased HIV promoter activity only in stimulated T cells. Three DNA clones containing HHV-6(GS) genomic fragments transactivated the HIV promoter in cotransfected T cells. A 21.4-kb DNA clone, pZVB70, showed the highest transactivating ability, while two other DNA fragments, pZVB10 (6.2 kb) and pZVH14 (8.7 kb), showed lower activity. One of these clones, pZVH14, activated the HIV promoter construct containing a mutation in the NFκB site. However, this mutated NFκB promoter was not transactivated during HHV-6(GS) infection or after cotransfection with pZVB70 or pZVB10. These data indicate that the NFκB sites of the HIV promoter are essential for its transactivation during HHV-6(GS) infection. By increasing HIV promoter activity in primary T lymphocytes, HHV-6 may consequently increase HIV replication, leading to an increase in the cytopathic effect on coinfecting human T cells.**

Human CD4<sup>+</sup> T lymphocytes are the primary target of the human immunodeficiency virus (HIV) (4, 20, 31). Infection with HIV results in the eventual development of AIDS (9, 10, 23). HIV replication can be shown in only a small percentage of T cells in vivo and seems to persist for long periods of time in a latent phase (2). Because the virus exists in a latent phase, infected individuals may not develop clinical manifestations of AIDS for years after the initial HIV infection (2, 15). The latent HIV virus can be reactivated by various agents such as antigen, mitogen, or cytokine stimulation (7, 21). A wide range of antigenic stimulation and viral infections have been demonstrated to activate the HIV promoter in various cell lines (13, 22, 40). However, it is not clear to what extent these events occur in vivo, and the mechanisms involved are not yet completely understood.

A new lymphotropic herpesvirus, human herpesvirus 6 (HHV-6), has recently been isolated from peripheral blood lymphocytes of patients with AIDS (35, 39), from patients with lymphoproliferative disorders (35), and from infants with exanthem subitum (34, 38). Immunological and molecular analyses have demonstrated that the new herpesvirus is distinct from other human herpesviruses (17, 18, 25). HHV-6 productively infects CD4<sup>+</sup> human T cells; however, the HHV-6 strains show variations in their ability to infect various T cells and T-cell lines (41). They also differ in antigenic properties (41) and exhibit DNA restriction site

heterogeneity (5). The prototype strain, HHV-6(GS), infected a number of T-cell lines as well as human B lymphocytes, glioblastoma cells, and megakaryocytic cells (26). In contrast, HHV-6(Z29), isolated from a Zairian AIDS patient, infected phytohemagglutinin-stimulated lymphocytes from cord blood or from adult peripheral blood but failed to replicate in unstimulated lymphocytes and certain T-cell lines (5). Recently, we have grown HHV-6(Z29) in the Molt-3 T-cell line, in which HHV-6(GS) failed to replicate.

HHV-6 infects many of the same target cells as HIV (26), and in vitro, HHV-6(GS) can also productively coinfect HIV-infected CD4<sup>+</sup> T cells (6, 24, 26). One study showed that this leads to accelerated cytopathic effects in comparison with HIV or HHV-6 infection alone (26). In addition, our previous studies have demonstrated that the HIV long terminal repeat (LTR) is activated in HHV-6-infected T-cell lines (14). Activation of the HIV LTR leads to increased HIV gene expression, which ultimately leads to a higher level of virus replication. These observations suggest that HHV-6 can act as a cofactor in the development and possibly in the progression of AIDS.

To study the activation of HIV by HHV-6 under more physiologic conditions, we have assessed whether or not HHV-6 infection of primary human T lymphocytes can activate the HIV promoter. Furthermore, the effects of specific antigenic stimulation in combination with infection by two strains of HHV-6 were examined. Results of the study indicate that HHV-6 strains GS and Z29 can activate the HIV promoter in primary human T lymphocytes and differed in their dependency on antigenic stimulation for transactivation. Transactivation by HHV-6(GS) involves the NFκB region of the HIV LTR. We have also identified three

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different cloned HHV-6(GS) DNA fragments that transactivate the HIV promoter after cotransfection into human T cells.

## MATERIALS AND METHODS

**DNA and plasmids.** The HIV chloramphenicol acetyltransferase (CAT) plasmid has been described in detail previously (19). Briefly, the construct contains both the 3' untranslated region and the repeat sequences of the HIV LTR aligned with the CAT gene. The -147 CAT plasmid was constructed by deleting a fragment of the HIV LTR at the *Ava*I site as described elsewhere (13). The -57 CAT plasmid was kindly provided by Joseph Sodroski (Harvard Medical School, Boston, Mass.) (33). The HIV LTR plasmid containing the mutated NF $\kappa$ B site was kindly provided by Gary Nabel (27). *Bam*HI plasmid libraries of the HHV-6 genome were generated as described previously (17).

**HHV-6 viral strains and preparation.** The human T-cell lines HSB-2 (ATCC CCL120.1 CCRF-HSB-2) and Molt-3 (ATCC CRL 1552) were grown in RPMI 1640 medium supplemented with fetal bovine serum and antibiotics. Two strains of HHV-6 were used in this study. HHV-6(GS) was a gift from R. C. Gallo, National Cancer Institute, Bethesda, Md.; HHV-6(Z29) was a gift from P. Pellet, Centers for Disease Control, Atlanta, Ga. HHV-6(GS) was grown in HSB-2 cells, and HHV-6(Z29) was grown in Molt-3 cells. Infected cells were collected 6 to 8 days postinfection when more than 80% of the cells were positive for viral antigens in an indirect immunofluorescence assay (IFA) by using HHV-6 specific monoclonal antibodies (3). These infected cells were frozen and thawed twice, and the 50% tissue culture infectivity dose was determined by procedures described previously (3, 41) on HSB-2 cells for HHV-6(GS) and Molt-3 cells for HHV-6(Z29).

**Human T lymphocytes.** Fresh mononuclear cells were isolated from the peripheral blood of purified protein derivative (PPD)-immune, normal human donors by using Hypaque-Ficoll (13). These cells ( $10^5$  per well) were then stimulated for 6 days with tuberculin PPD (5  $\mu$ l/ml) in 96-well round-bottom plates. Lymphoblasts were separated from small mononuclear cells and dead cells by using a 35 to 45% Percoll step gradient. The cells collected at the interface between 35 and 45% were judged to be >98% lymphoblasts by microscopic examination. By immunofluorescence, 85 to 89% of the cells were judged to be CD3+ (Ortho T3 antibody). These cells are referred to in this report as T-cell lymphoblasts and were used for transfections.

**Transfection and infection of T cells.** The T-cell lymphoblasts were transfected by a DEAE-dextran method (1). Briefly, 1  $\mu$ g of the appropriate DNA in the presence of 700  $\mu$ g of DEAE-dextran per ml was added to T-cell lymphoblasts or the T-cell line HSB-2 for 3 h at 37°C. The cells were then treated with dimethyl sulfoxide (10%) for exactly 1 min. After washing, the cells were placed in fresh RPMI 1640 medium containing 10% human serum. A portion of the transfected T cells was then infected with HHV-6(GS) or HHV-6(Z29) at a multiplicity of infection of 0.01 by incubating the T-cell pellet for 1 h at 37°C with cell lysates of HHV-6(GS) or HHV-6(Z29). In certain experiments, HHV-6(GS) DNA clones were cotransfected with the HIV promoter constructs into primary T lymphoblasts or the T-cell line HSB-2. The transfected and/or infected T cells ( $2 \times 10^4$  per well) were then added to either irradiated, adherent, autologous mononuclear cells ( $10^5$  per well) alone or cells that had been incubated overnight with tuberculin PPD (5

$\mu$ l/ml) in 96-well flat-bottom plates as described earlier (13). These cell populations are referred to as resting transfected T cells or stimulated transfected T cells, respectively. Five days later, the transfected T cells were collected, washed twice with phosphate-buffered saline, counted, and used in CAT assays. Since a background level of CAT activity is observed in fresh T-cell blasts as a result of antigenic stimulation, cells were collected 5 days after transfection in order to lower this background level of CAT activity.

**CAT assays.** Transfected T cells, collected after 5 days, were resuspended in 100  $\mu$ l of sonication buffer (0.25 M Tris HCl, pH 7.4). The cell number was adjusted to  $2 \times 10^6/100$   $\mu$ l so that the same number of cells was used in each CAT assay. Cellular extracts were prepared by a single freeze ( $-70^\circ\text{C}$ )-thaw ( $37^\circ\text{C}$ ) cycle and 1 min of sonication, followed by centrifugation to remove cell debris. CAT assays using [ $^{14}\text{C}$ ]chloramphenicol and thin-layer chromatography have been described in detail previously (12). CAT assays using [ $^3\text{H}$ ]acetyl coenzyme A ([ $^3\text{H}$ ]acetyl-CoA) have been described by Neuman et al. (30). Briefly, 50  $\mu$ l of the cell lysates, [ $^3\text{H}$ ]acetyl-CoA, and chloramphenicol was mixed in 100 mM Tris HCl, pH 8.0. An organic scintillation cocktail (Econofluor; Hewlett-Packard) was layered over this mixture, and the samples were incubated at 37°C. The samples were counted in a beta scintillation counter at hourly intervals after initiation of the assay. Units of CAT activity in each cell lysate were calculated according to a standard curve established with a CAT sample (Pharmacia) of known enzymatic activity.

## RESULTS

**HHV-6(GS) transactivates the HIV LTR promoter in primary human T lymphoblasts.** The primary T lymphoblasts used in this study have been described previously (13) and consist of approximately 85% CD3+, 59% CD4+, and 32% CD8+ cells. These lymphoblasts were transfected with the HIV CAT construct, containing the HIV promoter, by using DEAE-dextran. After transfection, a portion of the T cells was infected with HHV-6(GS). Five days after infection with HHV-6(GS), 30 to 40% of the cells reacted with HHV-6-specific monoclonal antibodies (3) by IFA. This result indicated that the transfected cells were productively infected by HHV-6(GS). None of the uninfected lymphocytes reacted with HHV-6-specific monoclonal antibodies, indicating the absence of endogenous HHV-6 virus. After 5 days in culture, lysates were prepared from the transfected cells and CAT activity was measured (Fig. 1). Lysates from transfected T cells without antigen (PPD) stimulation and without HHV-6 infection converted 9% of the chloramphenicol substrate to its acetylated forms, as shown by thin-layer chromatography (Fig. 1A). This lysate contained a total of 0.12 U of CAT activity (Fig. 1B and Table 1). Transfected T-cell blasts activated with antigen showed higher CAT activity than did unstimulated T cells. Lysates from these cells converted 79% of the chloramphenicol to its acetylated forms and contained a total of 0.46 U of CAT activity (Fig. 1C). This represents a fourfold increase in CAT activity after antigenic stimulation (Table 1). These experiments confirm our previous studies, which showed that antigen activation of primary T cells can activate the HIV promoter (13).

Infection of these transfected T cells with HHV-6(GS) resulted in transactivation of the HIV promoter in both resting and stimulated T cells (Fig. 1A and C). When lysates from unstimulated, GS-infected T-cell lymphoblasts were tested for CAT activity, there was a conversion of 75% of the

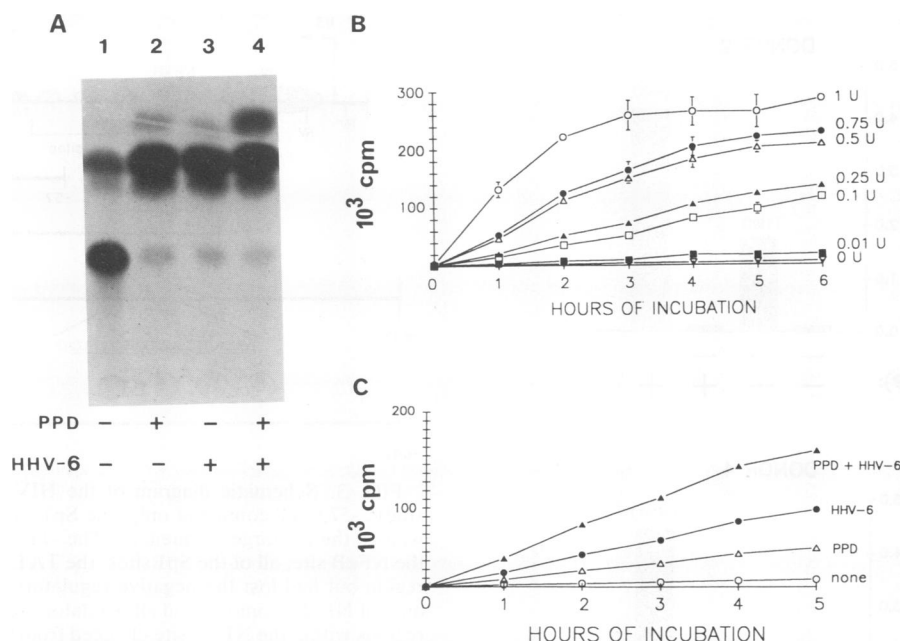


FIG. 1. CAT enzymatic activity from primary T-cell lysates. (A) Thin-layer chromatography of [<sup>14</sup>C]chloramphenicol reacted with cell lysates from stimulated T cells (PPD+) and resting T cells (PPD-) either infected with HHV-6 (+) or uninfected (-). (B) Standard curve of CAT enzymatic activity obtained by using a rapid method that utilizes [<sup>3</sup>H]acetyl-CoA. This standard curve is used to calculate the amount of CAT activity in cell lysates. As little as 0.01 U of enzymatic activity can be detected per lysate. (C) CAT activity measured in T-cell lysates by the rapid method.

substrate, corresponding to 0.64 U of CAT activity, while lysates from activated, infected T cells showed a 99% conversion of the substrate and 2.61 U of CAT activity (Fig. 1A and Table 1). These values represent 5- and 22-fold increases in CAT activity without and with antigenic stimulation, respectively. Since CAT assays with [<sup>3</sup>H]acetyl-CoA

are more quantitative and convenient (30), subsequent cell lysates were all measured by this rapid method.

These data show that infection of primary T lymphoblasts with HHV-6(GS) activates the HIV promoter in both resting and stimulated primary T cells. Although the activation in resting cells is not as pronounced (5- to 7-fold) as in antigen-activated T cells (22- to 25-fold), the data clearly demonstrate that HHV-6(GS) can infect unstimulated primary T cells and will activate the HIV promoter, which otherwise remains quiescent. Although activation of resting, primary T cells by HHV-6 may have also contributed to the low level of transactivation of the HIV promoter, this alone cannot account for all of the transactivation activity observed.

**Transactivation of the HIV promoter in antigen-activated primary human T lymphocytes by HHV-6(Z29).** Soon after the GS strain of HHV-6 was identified, a new variant strain of HHV-6 was identified which was designated HHV-6(Z29) (5). HHV-6(Z29) is related to the GS strain both antigenically and genetically (17, 25, 41). Both strains infect human T lymphocytes but seem to have a preference for distinct subsets. These subsets are still not clearly defined. The Z29 strain can effectively infect the Molt-3 T-cell line but not HSB-2, while the GS strain infects HSB-2 but not Molt-3. Both HHV-6 strains can infect primary human phytohemagglutinin-stimulated blasts, but HHV-6(Z29) failed to replicate in unstimulated peripheral blood lymphocytes (41). In this study, primary T lymphoblasts specific for the antigen tuberculin PPD were transfected with the HIV CAT construct and then infected with HHV-6(Z29). After 6 days, the cells were collected and tested for infection with HHV-6(Z29) by IFA using cross-reactive HHV-6(GS) monoclonal antibodies (41). T lymphocytes stimulated with PPD during the HHV-6(Z29) infection had enlarged cells and were pos-

TABLE 1. Evidence that the enhancer region of the HIV LTR is required for HHV-6(GS) transactivation<sup>a</sup>

Promoter	T-cell donor <sup>b</sup>	CAT activity (total U in cell lysate)			
		Uninfected cells		HHV-6-infected cells	
		-	+	-	+
HIV CAT	1	0.12	0.45 (4) <sup>c</sup>	0.78 (7)	3.04 (25)
	2	0.12	0.45 (4)	0.64 (5)	2.61 (22)
-147 CAT	1	0.24	1.11 (5)	2.97 (12)	3.84 (16)
	2	0.19	1.08 (6)	2.08 (11)	3.24 (17)
	3	0.16	0.31 (2)	1.49 (9)	2.32 (15)
-57 CAT	1	<0.01	<0.01	0.05	0.05
	2	<0.01	<0.01	0.03	0.04
Mutant NFκB-CAT	2	<0.01	<0.01	<0.01	<0.01
	3	<0.01	<0.01	<0.01	<0.01

<sup>a</sup> Various promoters were transfected into primary T cells by using DEAE-dextran. The transfected cells were either cultured directly with autologous, irradiated mononuclear cells or infected with HHV-6(GS) and then added to feeder cells. Antigen was then added to appropriate cultures. Cells were incubated for 5 days, collected, and lysed, and CAT activity was measured. Transfected cells were infected with HHV-6(GS) at multiplicity of infection of 0.01. Some cell cultures were given the antigen PPD (+), while others were given medium (-).

<sup>b</sup> Cells from at least two different donors were transfected with each promoter. Cells from each donor were transfected twice.

<sup>c</sup> Values in parentheses represent the increase in CAT activity based on the enzymatic activity found in noninfected, resting T cells.

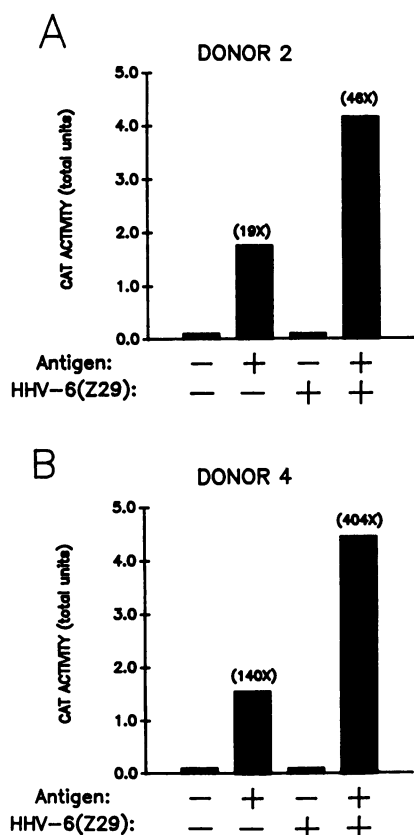


FIG. 2. Total CAT activity from resting (–) and stimulated (+) T cells either infected with HHV-6(Z29) (+) or left uninfected (–). Shown are representative results from two different donors; the values in parentheses show the increase in total CAT activity compared with the activity of unstimulated T lymphoblasts. The total units of CAT activity in unstimulated T cells are 0.09 for donor 2 and 0.011 for donor 4.

itive for viral antigens. However, T cells infected with HHV-6(Z29) in the absence of antigen stimulation showed no viral antigen-positive cells.

After 6 days of culture, transfected T lymphocytes were collected, washed, and lysed, and the CAT activity was measured from the cell lysates. The results showed that the HIV CAT promoter was activated only in HHV-6(Z29)-infected human T lymphocytes that had been stimulated with antigen. This was explained by the finding that productive infections of T lymphocytes with HHV-6(Z29), as determined by IFA with HHV-6-specific antibodies, occurred only in cells stimulated by antigen. Unstimulated cells did not support viral infection, nor did they demonstrate HIV CAT promoter activation (0.09 U of CAT activity in donor 2; 0.011 U of CAT activity in donor 4). As noted above, in primary T cells not infected with HHV-6(Z29), the HIV promoter was activated by antigenic stimulation (19-fold in donor 2, with 1.75 U of CAT activity; 140-fold in donor 4, with 1.54 U of CAT activity) (Fig. 2). When the transfected T cells were infected with HHV-6(Z29) in the absence of antigen, no increase in CAT activity was observed (0.97-fold in donor 2, with 0.087 U of CAT activity; 0.7-fold in donor 4, with 0.008 U of CAT activity). However, when the transfected T cells were infected with HHV-6(Z29) and activated with PPD, a 46- to 404-fold increase in total CAT activity and

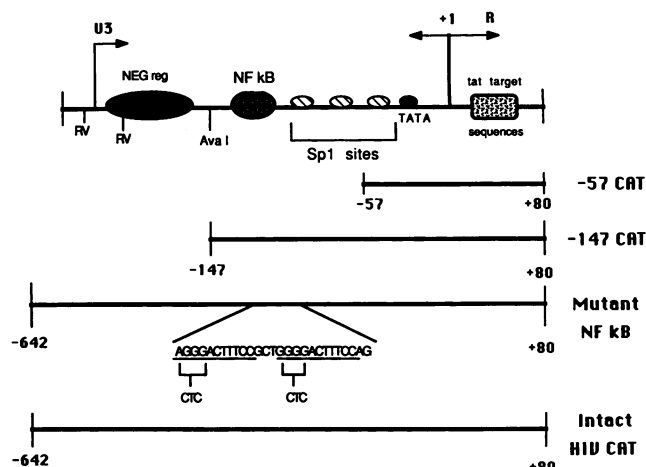


FIG. 3. Schematic diagram of the HIV promoters used in this study. -57 CAT contained only one Sp1 site and the TATA box as well as the *tat* target sequences. The -147 CAT plasmid contained the NFκB site, all of the Sp1 sites, the TATA box, and the *tat* target region but had lost the negative regulatory region (NEG reg). The mutant NFκB promoter had all regulatory regions intact but had two regions within the NFκB site changed from GGG to TCT. The intact HIV CAT promoter had no sequence changed or deleted.

3-fold increase in activity over antigenic stimulation alone were noted (4.18 U of total CAT activity in donor 2; 4.44 U of total CAT activity in donor 4). These data indicate that HHV-6(Z29) transactivated the HIV LTR in antigen-stimulated, primary, human T lymphocytes and that transactivation by HHV-6(Z29) requires productive infection of primary T lymphocytes.

**Transactivation of the HIV promoter in human T lymphocytes by HHV-6(GS) depends on the presence of a functional NFκB enhancer site.** Earlier studies on the activation of the HIV promoter in primary human T lymphocytes showed that activation of the HIV promoter was influenced by the NFκB enhancer region as well as an upstream region that exerts negative regulation (13). Several different constructs of the HIV promoter, described in Fig. 3, were transfected into PPD-specific human T lymphoblasts. The HIV promoter designated -57 CAT contains one of the Sp1 sites as well as the TATA site. This promoter has been shown to be active in other cell types (COS-7; data not shown), but little or no promoter activity has ever been observed in primary T lymphocytes (13) (Table 1). The promoter designated -147 CAT retained the NFκB binding region as well as the three Sp1 sites and the TATA box (Fig. 3). This promoter does not have the negative regulatory region, and therefore the CAT activity expressed after transfection into primary T lymphoblasts is somewhat higher than with the intact promoter (Table 1). T lymphoblasts transfected with -147 CAT and then infected with HHV-6(GS) consistently showed evidence of transactivation (9- to 17-fold increase in CAT activity). This finding indicates that the negative regulatory region is not primarily involved in the increased promoter activity due to HHV-6(GS) infection. These results also show that the DNA sequences responsible for HHV-6(GS) transactivation must be within the -147 to -57 region. To further locate the specific promoter region responsible for HHV-6 transactivation, the HIV LTR plasmid containing a mutated NFκB CAT promoter was transfected into primary T lymphocytes. This promoter contained two sequences

TABLE 2. Evidence that HHV-6(GS) DNA plasmids can transactivate the HIV promoter<sup>a</sup>

		CAT activity (total U in cell lysate)			
		HIV-CAT		HIV-CAT + HHV-6(GS) plasmid	
T-cell donor	Antigenic stimulation		Plasmid	Antigenic stimulation	
	-	+		-	+
2	0.04	1.8 (45) <sup>b</sup>	pZVB70	4.05 (101)	10.84 (271)
3	0.12	0.26 (2)		4.54 (38)	10.88 (91)
3	0.15	1.95 (13)		3.85 (26)	11.55 (77)
2	0.1	1.24 (12)	pZVH14	1.77 (18)	2.58 (26)
3	0.24	1.95 (8)		2.37 (10)	3.14 (13)
3	0.19	1.46 (8)		1.77 (9)	2.87 (15)
1	0.02	1.35 (68)	pZVB10	0.26 (13)	1.68 (84)
4	0.01	1.54 (154)		0.35 (35)	1.83 (183)
1	0.02	1.7 (85)	pZVB9	0.02	1.12 (56)
3	0.24	1.95 (8)		0.17	1.51 (6)
3	0.38	1.91 (5)	pZVB15	0.07	2.79 (7)
4	0.01	1.54 (154)		0.05 (5)	1.87 (187)
2	0.1	1.24 (12)	pZVB43	0.17 (2)	1.37 (14)
3	0.15	1.95 (13)		0.14 (1)	1.63 (11)
3	0.19	1.46 (8)		0.18 (1)	1.69 (9)

<sup>a</sup> Plasmids containing HHV-6(GS) DNA were cotransfected with HIV CAT into primary T cells. CAT activity was measured after 5 days from cell lysate. Antigen activation of transfected cells was as described for Table 1.

<sup>b</sup> See Table 1, footnote c.

within the NFκB sites that had been changed from GGG to CTC (Fig. 3), changes that in the NFκB site eliminate the activation of the HIV promoter by the NFκB protein (27). This mutation in the NFκB sites abolished the HIV promoter activity induced in primary T lymphoblasts both by antigen and by HHV-6(GS) infection. Although no CAT activity could be detected in the lysates from these cells, IFA indicated that the population of T cells transfected with the mutant NFκB promoter and infected with HHV-6(GS) contained enlarged cells highly positive for HHV-6(GS) proteins (data not shown). This result showed that the transfected T cells were productively infected with HHV-6(GS) and suggests that HHV-6(GS) requires the NFκB region for transactivation of the HIV promoter.

At least three different DNA segments cloned from the HHV-6(GS) genome can transactivate the HIV promoter when cotransfected into primary T lymphocytes. The HHV-6(GS) genome was digested with the restriction enzyme *Bam*HI or *Hind*III, and the resulting fragments were ligated into the Bluescript vector (17). Six different HHV-6(GS) DNA fragments representing about 40% of the total viral genome and varying in length from 3.3 to 21.4 kb (17) were used in these studies. Each HHV-6(GS) fragment was cotransfected with HIV CAT into the primary T-cell populations, and CAT activity was measured in cell lysates 5 days later. The largest fragment, pZVB70 (21.4 kb), showed a dramatic transactivating effect on the HIV promoter (Table 2). In the absence of antigen, this fragment increased HIV promoter activity 26 to 101 times over the activity expressed in unstimulated T cells. When the cells were stimulated with antigen, there was a 77- to 271-fold increase in total CAT activity, which was 6- to 42-fold over the antigenic stimulation alone. It was the major DNA fragment from HHV-6(GS) that showed this level of activity, and as such it was designated a high transactivator of the HIV promoter.

Two other HHV-6(GS) fragments, designated pZVH14 and pZVB10, marginally transactivated the HIV promoter

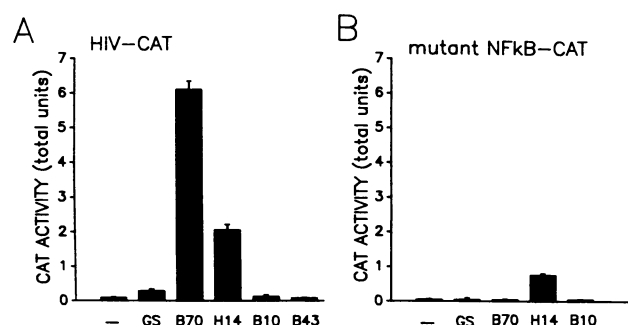


FIG. 4. Total CAT activity from transfected HSB-2 cells. (A) HSB-2 cells were transfected with HIV CAT only (-) and then infected with HHV-6(GS). Cells were also cotransfected with HIV CAT and various HHV-6 cloned DNA fragments: pZVB70 (B70), pZVH14 (H14), pZVB10 (B10), and pZVB43 (B43). (B) HSB-2 cells were also transfected with mutant NFκB CAT plasmid. Some of the cells were infected with HHV-6(GS), while others were left uninfected (-). A portion of the cells were cotransfected with various HHV-6 DNA clones as described above.

(Table 2) when expressed in the absence of antigen stimulation. The DNA clone pZVH14 contained a 8.7-kb fragment from HHV-6(GS), while clone pZVB10 contained a 6.2-kb fragment. Since these HHV-6(GS) DNA clones showed only low-level activation of HIV CAT in the absence of antigen and promoter activity in antigen-stimulated cells increased only slightly, we have designated these clones low transactivators. The highest transactivation was observed when cotransfected T lymphocytes were stimulated with antigen, suggesting an additive effect of antigenic stimulation and the HHV-6(GS) gene product(s) on HIV promoter activity. Table 2 also shows data for three fragments cloned from HHV-6(GS) that failed to transactivate the HIV promoter. These DNA clones were designated pZVB9 (11.8-kb fragment), pZVB43 (8.3-kb fragment), and pZVB15 (3.3-kb fragment). From these results, it can be concluded that HHV-6 contains at least three different gene segments that can transactivate the HIV promoter.

To confirm the data observed in primary T lymphocytes, these fragments were also cotransfected with HIV CAT into the human T-cell line HSB-2 (Fig. 4A). As previously demonstrated (14), infection of the transfected HSB-2 cells with HHV-6(GS) resulted in an increase in CAT activity (threefold), indicating that the HIV promoter was also transactivated in a T-cell line. Cotransfection of HHV-6(GS) DNA clone pZVB70 and HIV CAT resulted in a dramatic increase in CAT activity (58-fold; Fig. 4A). Plasmid pZVB70 transactivated HIV CAT to a much greater extent than did HHV-6(GS). It was also observed that plasmid pZVH14, when cotransfected with HIV CAT into the HSB-2 T-cell line, activated promoter activity to a higher level than did HHV-6(GS). The CAT activity in these samples increased 30-fold over the activity of HSB-2 cells transfected with HIV CAT alone. This finding suggests that during HHV-6(GS) infection, the gene products from these DNA segments are under some type of regulation that is lost when the genes are cloned into plasmids. Cotransfection of HSB-2 cells with two other HHV-6 fragments, pZVB10 and pZVB43, did not show any transactivation of HIV CAT. These data confirm our observation in primary T lymphocytes, that the gene segments contained in plasmids pZVB70 and pZVH14 transactivated the HIV promoter; however, unlike the situation

TABLE 3. Transactivation of the mutant NF $\kappa$ B promoter with HHV-6(GS) DNA clones<sup>a</sup>

CAT activity (total U)					
T-cell donor	Mutant NF $\kappa$ B		Mutant NF $\kappa$ B + HHV-6(GS) plasmid		
	Antigen stimulation		Plasmid	Antigenic stimulation	
	–	+		–	+
3	<0.01	<0.01	pZVH14	0.07 (7) <sup>b</sup>	0.07 (7)
2	<0.01	<0.01		0.04 (4)	0.06 (6)
2	<0.01	<0.01	pZVB70	<0.01	<0.01
3	<0.01	<0.01		<0.01	<0.01
2	<0.01	<0.01	pZVB10	<0.01	<0.01
4	<0.01	<0.01		<0.01	<0.01

<sup>a</sup> HHV-6(GS) DNA clones were cotransfected with the mutant NF $\kappa$ B promoter (see Fig. 3) into primary T cells. Cells were incubated for 5 days as described for Table 1. Total CAT activity was measured in cell lysates.

<sup>b</sup> See Table 1, footnote c.

for primary T cells, pZVB10 failed to activate the HIV promoter in HSB-2 cells.

To determine whether mutations in the NF $\kappa$ B site of the HIV promoter affected the transactivation by cloned HHV-6(GS) DNA fragments, the three transactivating fragments were cotransfected into primary T cells with the promoter containing the NF $\kappa$ B mutation. The resulting promoter activities are shown in Table 3. Mutation in the NF $\kappa$ B site completely abolished the ability of the pZVB70 and pZVB10 clones to transactivate. However, with the pZVH14 clone, a slight but consistent transactivation was observed (four- to sevenfold) that was less than the transactivation seen with the intact HIV promoter. The transactivation of the mutated NF $\kappa$ B promoter by pZVH14 was not dependent on antigenic stimulation of the T cells, since it was not enhanced by the presence of antigen. Similar results were observed when these cloned DNA fragments were cotransfected with the mutated NF $\kappa$ B promoter into HSB-2 cells (Fig. 4B). The two DNA clones, pZVB70 and pZVB10, failed to transactivate the mutated NF $\kappa$ B promoter, but pZVH14 showed slight transactivation (8- to 10-fold). This finding confirmed the results in primary T lymphocytes. Together, these data suggest that the transactivating gene(s) included within the pZVH14 clone may not totally depend on an intact NF $\kappa$ B site but may also use other target DNA region(s) in the promoter.

## DISCUSSION

Our studies demonstrate that two strains of HHV-6, GS and Z29, can infect and activate the HIV promoter in primary, human T lymphocytes. However, these two strains of HHV-6 showed distinct differences in ability to transactivate the HIV promoter, related to the ability of the two strains to productively infect resting T lymphocytes. HHV-6(GS) infected both stimulated and unstimulated human T cells, while HHV-6(Z29) infected only stimulated T cells. This observation confirms the results of Wyatt et al. (41), who showed that HHV-6(Z29) failed to replicate in nonactivated peripheral blood lymphocytes. HHV-6(Z29) was unable to infect resting T cells, and no transactivation of the HIV promoter was observed. However, HHV-6(GS) transactivated the HIV promoter even in resting T cells. These data suggest that active replication of HHV-6(Z29) and subsequent production of its gene products are required for transactivation of the HIV promoter. It is also possible that

HHV-6(Z29) requires essential cellular elements that are provided by antigenic stimulation of T cells for its replication, and thus the activation of the HIV promoter by Z29 may require cellular cooperation. Ensoli et al. (8) have previously shown that HHV-6(GS) infection of HIV-infected T cells increases the steady-state level of HIV mRNA that parallels CAT enzymatic activity. It is quite possible that similar mechanisms are responsible for the increase in CAT enzymatic activity observed here. Studies are currently under way to examine the mechanism of action by the cloned gene fragments.

Certain difficulties are inherent in these types of experiments. We have observed differences in HIV promoter activation between different donors and even from the same donor when cells were donated at different times (6 months later). Without HHV-6 infection or antigenic stimulation, the background CAT activity ranged between 0.01 and 0.38 total U of enzyme activity. Because the increase in promoter activity is based on the background activity between donors or experiments, this parameter cannot be used to compare promoter activity but must be used to measure transactivation within a single experiment. This was not expected, since these experiments require many steps of long incubation periods. Because of the large number of peripheral blood mononuclear cells needed for each experiment ( $>100 \times 10^6$ ), donors were only bled every 6 weeks. However, each transfection was done at least twice on two different healthy adult human donors. Therefore, when transactivation was observed consistently in all experiments, we concluded that there was activation of the HIV promoter in these situations.

A number of experiments were performed to identify the region of the HIV promoter required for transactivation by HHV-6(GS). In earlier studies, several deletions or mutations in the HIV promoter were used to determine which promoter regions were essential for control of the promoter activity during primary in vitro T-cell stimulation (13). These altered promoters were also used in this study, and the results showed that the NF $\kappa$ B region was essential for transactivation during HHV-6(GS) infection. Thus, an HIV promoter containing mutated sequences within the NF $\kappa$ B region was no longer activated by HHV-6(GS) infection. It is not known whether HHV-6(GS) directly activates the NF $\kappa$ B cellular protein or, alternatively, a HHV-6(GS) gene product(s) binds directly to the NF $\kappa$ B region. However, these results to indicate that this particular promoter region seems to be essential for HHV-6(GS) transactivation during an active infection. Similar results were observed when the human T-cell line HSB-2 was transfected with the mutated NF $\kappa$ B promoter and then infected with HHV-6(GS). HHV-6(GS) is not unique in using the NF $\kappa$ B site, since it has been noted that other herpesviruses, such as herpes simplex virus type 1, cytomegalovirus, and Epstein-Barr virus, also act on the NF $\kappa$ B region of the HIV promoter (22).

The genome of HHV-6(GS) has been estimated to be about 170 kb in length (17, 18), which should code for more than 70 proteins. A number of DNA cloned fragments from the GS genome were examined in this study to determine whether they code for products that transactivate the HIV promoter. Several HHV-6(GS) DNA clones, comprising about 40% of the viral genome, were individually cotransfected with HIV CAT into primary human T lymphocytes. Three different non-cross-hybridizing DNA clones transactivated the HIV promoter, and at present it is not known whether other HHV-6 DNA fragments are also able to transactivate the HIV promoter. The DNA clone showing the highest level of transactivation (pZVB70) contained 21.4



kb of HHV-6(GS) DNA. It is possible that more than one gene product expressed from this plasmid is responsible for the high level of transactivating ability of this plasmid. Since these fragments were not inserted into a eukaryotic expression vector, their expression requires endogenous promoter activity. It can thus be inferred from these data that at least some of the HHV-6 promoters must be active in both stimulated and unstimulated primary T cells. Two other plasmids containing HHV-6(GS) fragments also transactivated the HIV promoter in primary T cells. These plasmids were pZVH14, which contained an 8.7-kb viral fragment, and pZVB10, which contained a 6.2-kb fragment. To determine the nature of the open reading frames present in these fragments, the DNA was sequenced (data not shown). From this information, several genes have been identified and are currently being subcloned and tested for transactivating ability on the HIV promoter in human T cells.

Although the pZVH14 clone was less active than pZVB70, it transactivated HIV CAT in the T-cell line HSB-2 and in primary T cells. Of interest was the observation that this fragment, pZVH14, was unique in transactivating the mutant NF $\kappa$ B promoter even in the absence of antigenic stimulation. No such activity was seen after GS infection of T cells or transfection of primary T cells with the two transactivating DNA fragments from GS, pZVB70 and pZVB10. These data also suggest that the gene products expressed from pZVH14 are able to act on regions other than the NF $\kappa$ B site, independent of antigenic stimulation. This also implies that gene expression from this DNA fragment is possible even without overt cellular stimulation. Since transactivation of the mutant NF $\kappa$ B was not observed during an actual infection with HHV-6(GS), the gene(s) contained within the pZVH14 DNA fragment must be under some type of regulatory control. A recent study has shown that the pZVH14 genomic fragment also has oncogenic properties (32). At present it is not known whether the gene product(s) induce transformation in NIH 3T3 cells is the same product(s) responsible for transactivation of the HIV promoter.

The immunodeficiency disease AIDS is a chronic disease caused by the human retrovirus HIV. The time from HIV infection to the clinical manifestation of AIDS can range from 2 months to more than a decade (2, 15, 16). An important determinant of the length of this latency period is the rate of HIV replication. Factors that accelerate HIV replication increase the progression of the disease. After HIV infection of human T cells in vitro, the virus undergoes a form of latency until activated by mitogens or specific antigens (42). In vitro stimulation of T cells increases HIV promoter activity (13, 40), which can terminate HIV latency. Thus, activation of the HIV promoter can be a factor in the length of the latency period. On the basis of their ability to activate the HIV promoter, other DNA viruses, such as herpes simplex virus type 1, cytomegalovirus, Epstein-Barr virus (22), papovaviruses (11), adenoviruses (28), and hepatitis B virus (36), have been implicated as possible cofactors in AIDS. Recent studies have shown that the herpesvirus cytomegalovirus enhances the replication of HIV in coinfecting cells (37), and cells productively infected with both CMV and HIV have been found in the brains of AIDS patients (29). These studies indicate that it is possible for DNA viruses, especially herpesviruses, to activate HIV even in vivo.

HHV-6 infects primarily CD4<sup>+</sup> human T cells, the same cells infected with HIV. Several investigators have shown that peripheral blood mononuclear cells can be productively coinfecting with HHV-6 and HIV (6, 24, 26). In fact, many of

the initial HHV-6 strains were isolated from individuals suffering from AIDS (25, 35, 39). Therefore, it seems likely that coinfection of human T cells with HHV-6 and HIV could accelerate the immunodeficiency disease caused by HIV. Ensoli et al. (8) showed that T-cell line HSB-2 could be productively coinfecting with HHV-6 and HIV, resulting in accelerated cell death and increased HIV transcription. In the present study, the HIV promoter was activated in HHV-6-infected, primary human T lymphoblasts. In contrast, recent reports have shown that coinfection of peripheral blood mononuclear cells with HHV-6 and HIV suppressed HIV replication (6, 24). The differences between these studies may be due to the nature of the cells that were infected, dosage of virus, and time of infection. In the present study, 85 to 89% of the transfected cells were human T lymphoblasts, whereas the other studies (6, 24) used stimulated peripheral blood mononuclear cells which contained a variety of cell types. It has been demonstrated that HHV-6 infects numerous cell types present in peripheral blood mononuclear cells, including monocytes and B cells (25, 26, 39). Since the mechanism of HIV inhibition by HHV-6 in these studies is not known, it still remains possible that HHV-6 infection of cell types other than T cells contributes to suppression of HIV replication through cytokine production.

This study has demonstrated that HHV-6 gene products can activate the HIV promoter in primary human T lymphoblasts. Therefore, an active HHV-6 infection of human T cells can activate an otherwise quiescent HIV promoter. This promoter activation can lead to an increased HIV replication and consequently enhances T-cell death. In addition, the replication of HHV-6 itself is cytopathic for T cells, which would start a cycle of increased T-cell death. By this proposed mechanism, an active HHV-6 infection would accelerate the progression of AIDS.

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