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Identification and Characterization of a Human Herpesvirus 6 Gene Segment That *trans* Activates the Human Immunodeficiency Virus Type 1 Promoter

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Human herpesvirus 6 (HHV-6) is a lymphotropic herpesvirus, and in vitro, HHV-6 can productively infect many of the same cell types as can human immunodeficiency virus (HIV). Coinfection by both viruses in vitro can lead to both activation of the HIV promoter and acceleration of cytopathic effects. We have previously demonstrated that a large, 22.25-kb cloned HHV-6 fragment, pZVB70, can *trans* activate HIV promoter expression in vitro. In this study, we show that the pZVB70 fragment can *trans* activate the HIV promoter in human T-cell lines as well as in the monkey kidney cell line CV-1. The pZVB70 insert was digested with various restriction enzymes, and individual fragments were transfected into cells to test for their ability to *trans* activate the HIV promoter. By this method, we have identified a 1.8-kb subfragment, B701, that is involved in *trans* activation. Sequence analyses show that B701 potentially encodes a 143-amino-acid protein. This protein shares no homology with other herpesvirus proteins, such as ICP0 and ICP4, that have been shown to *trans* activate the HIV promoter. However, it shows weak sequence homology with the gene products encoded by the cytomegalovirus early US22 gene family, suggesting that the putative B701 protein may be an HHV-6 early regulatory protein. The 143-amino-acid coding sequence of B701 was cloned by polymerase chain reaction, and transfection of this construct into cells activated HIV promoter expression. The target site on the HIV promoter for the putative B701 protein is mapped to the NF- κ B binding site. Our results suggest that the putative B701 protein may function by directly binding to the NF- κ B site or may involve cellular factors, such as NF- κ B, either directly or indirectly.

Human immunodeficiency virus (HIV) is the etiologic agent for AIDS. Infection by HIV can lead to either acute or chronic infections, and activation signals are necessary to establish a productive infection (8). Latent infection can persist for years after the initial HIV infection, which can be stimulated into acute phase by various cofactors (3). These cofactors probably play a very important role in the pathogenesis of HIV infection and in the clinical manifestation of AIDS. These cofactors could be of nonviral origin, such as lymphokines (9), mitogens, or antigens (12, 33). In addition, exogenous viral infection, such as by herpes simplex virus (HSV) (22, 23), adenovirus (28), and hepatitis B virus (31), have all been demonstrated to stimulate HIV replication in vitro. It has also been shown that viral infections can lead to the activation of HIV long terminal repeat (LTR)-directed viral gene expression (10, 22, 32). The mechanism of activation is not completely clear, but it may involve viral gene products, such as the immediate-early gene products of HSV-1 (1) and cytomegalovirus (6). It is possible that these viral gene products interact directly with the HIV LTR, or indirectly via cellular factors such as NF- κ B, in the *trans* activation of HIV (10).

Among the herpesviruses that have been shown to *trans* activate HIV expression, the newly identified Human herpesvirus 6 (HHV-6) seems to be most interesting. HHV-6 has been isolated from AIDS patients (30, 35), from patients with lymphoproliferative disorders (30), and from children

with exanthem subitum (34). HHV-6 infection among humans is very common, and most individuals are seropositive for HHV-6 (15). In vitro, HHV-6 can productively infect many of the same cell types as can HIV, particularly CD4⁺ human T cells, and coinfection of T cells with HIV and HHV-6 leads to accelerated cytopathic effects (20). Recently, it has been shown that infection of CD8⁺ cells by HHV-6 leads to surface expression of the CD4 molecule, thus rendering these cells susceptible to HIV infection (21). In contrast, other recent studies demonstrated that coinfection of peripheral blood mononuclear cells with HHV-6 and HIV suppressed HIV replication (4, 18). The differences between these studies may be due to the nature of the cells that were infected, dosage of virus used, and the time of infection. Therefore, it is important to isolate the specific HHV-6 genes that may be involved in either activation or suppression of HIV.

We have demonstrated previously that in vitro infection with HHV-6 strains GS and Z-29 can activate HIV LTR expression in human T-cell lines (13) and in a heterogeneous population of antigen-specific primary human T lymphocytes (14). Activation of the HIV LTR by HHV-6 in normal human T lymphocytes potentially reflects an in vivo situation, where there may be additive effects on *trans* activation by HHV-6 and stimulation with specific antigen. Taken together, these results suggest that HHV-6 can potentially play an important role in vivo as a cofactor in HIV infection and development of AIDS.

To gain further insight into the mechanism of HHV-6 *trans* activation of the HIV LTR, it is important to identify the HHV-6 gene(s) that is involved. The genome of HHV-6 is

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about 170 kb in size, and very little is known about the identities and functions of its gene products (16). We have previously demonstrated that a 22.25-kb HHV-6 (GS) gene segment (pZVB70) can *trans* activate the HIV LTR after cotransfection into human T-cell lines (14). Here we report the identification and characterization of a 1.8-kb gene segment from the 22.25-kb pZVB70 fragment that can *trans* activate the HIV LTR. Sequence analyses show that the putative *trans*-acting protein is 143 amino acids in size and shares no homology with other herpesvirus gene products shown to *trans* activate the HIV LTR.

MATERIALS AND METHODS

Cell lines. The human T-cell line HSB-2 (ATCC CCL 120.1) and the African green monkey kidney cell line CV-1 (ATCC CCL 70) were used. T cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, while CV-1 cells were maintained in Dulbecco modified Eagle medium with 10% fetal calf serum.

DNA and plasmids. Construction of various HIV-chloramphenicol acetyltransferase (CAT) plasmids was previously described in detail (17). Briefly, the HIV-CAT construct contains both the 3' untranslated regions and the repeat sequences of the HIV LTR ligated to the CAT gene. The -29 CAT plasmid was constructed by deleting all regulatory elements on the HIV LTR except for the TATA box and the TAR region. The -57 CAT plasmid was kindly provided by Joseph Sodroski (Harvard Medical School, Boston, Mass.) (29). The -147 CAT plasmid was constructed by deleting a fragment of the HIV LTR at the *Ava*I site as described elsewhere (12). The HIV LTR plasmid containing the mutated NF- κ B site was kindly provided by Gary Nabel (24). Construction of HHV-6 pZVB70 and pZVB9 plasmids containing *Bam*HI fragments of the HHV-6 genome was described previously (14).

Transfections. HSB-2 cells were transfected by the DEAE-dextran method (2). Briefly, 1 μ g of DNA, suspended in 700 μ g of DEAE-dextran per ml, was added to HSB-2 cells. Cells were then incubated for 2 h at 37°C. The cells were then treated with dimethyl sulfoxide (1%) for exactly 90 s. After being washed with RPMI 1640 without serum, the cells were placed in fresh RPMI 1640 medium containing 10% fetal calf serum. Two days later, the transfected cells were harvested, washed twice with phosphate-buffered saline (PBS), and then used for CAT assays.

CV-1 cells were transfected by using standard calcium phosphate precipitation procedures (11). Briefly, 0.5×10^6 to 1×10^6 cells were incubated with 2 μ g of plasmid DNA suspended in calcium phosphate for 8 h and then treated with 15% glycerol for 3 min. Cells were then washed with PBS and cultured in fresh medium. After 2 days, transfected cells were harvested for CAT assays.

CAT assays. CAT assays were performed by using [3 H]acetyl coenzyme A ([3 H]acetyl-CoA). Harvested cells were resuspended in 100 μ l of sonication buffer (0.25 M Tris-HCl, pH 7.8). Cellular extracts were prepared by freeze-thawing once, sonicating for 1 min, and briefly centrifuging to pellet cell debris. HSB-2 cell lysates were used for CAT assay directly, whereas CV-1 cell lysates were heat treated at 65°C for 5 min before CAT analysis. Protein concentrations for each sample were determined by the BCA protein assay (Pierce Chemical Co.). All samples were corrected for protein concentration variations before use in CAT assays. Procedures for the CAT assay using [3 H]acetyl-CoA were previously described (25). Briefly, 50 μ l of cell

lysate was mixed with 200 μ l of reaction mixture (130 μ l of Tris-HCl [pH 7.8], 50 μ l of 50 mM chloramphenicol, 1 μ Ci of [3 H]acetyl-CoA, 19 μ l of 4 mM acetyl-CoA). Samples were then overlaid with organic scintillation fluid and incubated at 37°C; counts were taken at 0, 1, 2, 3, 4, and 5 h after initiation of the assay. Units of CAT activity for each lysate were calculated by extrapolation according to a standard curve established with known CAT enzymatic activities.

DNA sequencing and analysis. DNA sequence analyses were performed by the Sequenase (U.S. Biochemical Corp., Cleveland, Ohio) method. Sequence data were analyzed by using the SAP program (Universal Biotechnology, Inc., Rockville, Md.) and by the IBI-Pustell sequence analysis program. Amino acid homology analysis was conducted by using the FASTA program developed by Lipman and Pearson (19).

Nucleotide sequence accession number. The sequence data presented in this report have been submitted to the GenBank and EMBL data bases under accession number M81789.

RESULTS

HHV-6 (GS) genomic fragment (pZVB70) can *trans* activate the HIV LTR in CV-1 cells. We have demonstrated previously that the HIV LTR can be *trans* activated by HHV-6 infection in human T-cell lines and in primary human T cells. Furthermore, the 22.25-kb (pZVB70) HHV-6 genomic segment was shown to *trans* activate the HIV LTR when cotransfected into human T cells (14). To determine whether HHV-6 *trans* activation of the HIV LTR is cell type specific, similar transfection studies were carried out in CV-1 cells. These cells were chosen since various studies, including our own, have shown that CV-1 cells can be consistently transfected with the plasmid containing the HIV LTR and provide a good system with which to study *trans* activation of the HIV LTR by various cofactors (27). CV-1 cells were cotransfected with HIV-CAT and pZVB70 DNA by the calcium phosphate precipitation method. At 48 h after transfection, cells were harvested and CAT activity was measured. All CAT assays were done with [3 H]acetyl-CoA because this method is quantitative, reproducible, and convenient (25). Assays were carried out for 5 h, and CAT activities were measured hourly (Fig. 1). Increases in CAT activity were calculated by using a standard curve of known units of CAT enzymatic activities. Cotransfection of HIV-CAT with pZVB70 resulted in a more than 10-fold increase in CAT activity, and the *trans* activation was very consistent. For a negative control, other cloned HHV-6 gene segments were cotransfected into CV-1 cells along with HIV-CAT. Figure 1 shows the results of such a study using the 11.8-kb fragment (pZVB9). No *trans* activation of the HIV LTR was observed with this or other control fragments. These data demonstrate that the *trans* activation of the HIV LTR by the HHV-6 DNA fragment pZVB70 is not just T-cell specific.

A functional NF- κ B enhancer element is necessary for *trans* activation of the HIV LTR by pZVB70 in CV-1 cells. We have previously demonstrated that *trans* activation of the HIV LTR by HHV-6 (GS) and the pZVB70 fragment is dependent on the presence of a functional NF- κ B sequence, since an NF- κ B mutant promoter was not *trans* activated by HHV-6 (13, 14). To study the effects of the enhancer element on the HIV LTR and to determine whether *trans* activation of HIV-CAT by pZVB70 in CV-1 cells also involves NF- κ B, transfection studies were carried out by using various HIV promoter constructs (Fig. 2). Different constructs of the HIV LTR were cotransfected along with pZVB70 into CV-1 cells

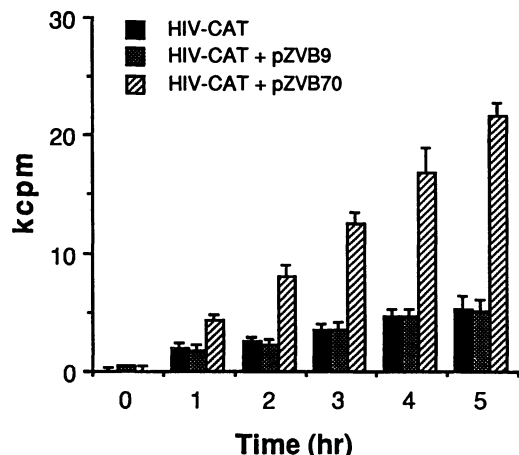


FIG. 1. CAT enzymatic activity in CV-1 cells. CV-1 cells were either transfected with HIV-CAT alone or cotransfected with HIV-CAT and different HHV-6 plasmids. CAT activities were quantitated by using [³H]acetyl-CoA, and incorporated radioactivities were counted hourly. Data represent the means of at least three independent experiments.

(Fig. 3). The promoter constructs -29 CAT and -57 CAT, containing deleted NF- κ B binding regions, lost their ability to be *trans* activated by pZVB70. Within the -147 CAT promoter construct, only the negative regulatory element has been deleted, and this construct can still be *trans* activated by pZVB70. The levels of activation were similar to those of the intact HIV-CAT. These results thus suggest that the target sequence may be located within the NF- κ B region. This possibility was further confirmed when an

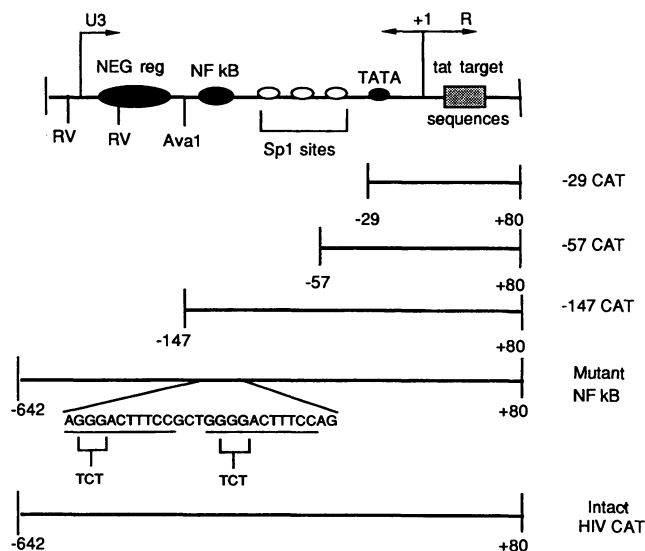


FIG. 2. Schematic diagram of various deleted HIV LTR-CAT constructs used in transfection studies. The -29 CAT plasmid represents a deletion of all upstream elements up to the TATA box. The -57 CAT plasmid contains only one Sp1 site and the TATA box. The -147 CAT plasmid contains the NF- κ B site, the Sp1 sites, the TATA box, and the *tat* target region; it has lost the negative regulatory element (NEG reg). The mutant NF- κ B promoter contains mutations in the NF- κ B regions (GGG changed to TCT). Intact HIV CAT represents the wild-type HIV LTR. RV, *EcoRV*.

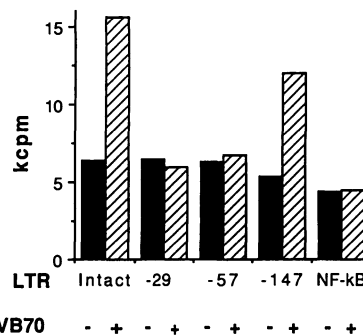


FIG. 3. CAT activities in CV-1 cells that were transfected with various deleted HIV-CAT constructs (as described in the legend to Fig. 2) either in the presence or in the absence of pZVB70.

NF- κ B mutant promoter was cotransfected along with pZVB70. This mutant promoter contains substitutions within each of the core enhancer elements which were implicated in binding of cellular NF- κ B. These substitutions eliminated activation of the HIV promoter by the NF- κ B protein (24). *trans* activation by pZVB70 in CV-1 cells was completely abolished when the NF- κ B mutant promoter was used. These results suggest that the NF- κ B sequence must be involved in *trans* activation by pZVB70 in CV-1 cells, similar to the results obtained earlier in human T cells (7, 13, 14).

Identification of a 3.8-kb fragment of pZVB70 which possesses *trans* activation function. The pZVB70 clone contains a 22.25-kb insert. Hence, it is necessary to further identify and characterize the specific gene(s) involved in *trans* activation. Various restriction enzymes were used to cut the pZVB70 DNA. The digested DNA were then transfected, along with HIV-CAT, into CV-1 cells and the human T-cell line HSB-2 to determine whether they have retained their *trans*-activating functions. Most restriction enzymes used eliminated the *trans*-activation function of pZVB70. However, digestion with several enzymes, such as *SalI*, did not affect its activity. Digestion of the pZVB70 22.25-kb insert by *SalI* gave five different smaller fragments of 1.16, 3.0, 3.8, 6.0, and 8.2 kb in size. The *SalI* restriction enzyme-cut sites of pZVB70 are shown schematically in Fig. 4A. When each *SalI* fragment was purified and cotransfected individually into CV-1 cells along with HIV-CAT to test for *trans* activation, only the 3.8-kb fragment *trans* activated HIV-CAT (Fig. 4B). The *trans*-activation level of the purified 3.8-kb fragment is slightly lower than that of intact pZVB70 (Fig. 4B). This difference may be due to differences in efficiency of transfection. Nevertheless, our results indicate that the 3.8-kb fragment contains the gene(s) responsible for the majority of the *trans*-activation activities observed with the pZVB70 fragment.

Sequence analyses of the 3.8-kb fragment. To locate the coding regions within the 3.8-kb fragment, the entire fragment was sequenced and analyzed for putative open reading frames (ORFs). Sequence analysis revealed three ORFs that can encode for proteins larger than 100 amino acids in size. These three ORFs, designated ORF-A, ORF-B, and ORF-C, are shown in Fig. 5. ORF-A initiates at nucleotide 1032 and terminates at nucleotide 1805; its first methionine is located at nucleotide 1378, and it can potentially encode a protein of 143 amino acids. ORF-B initiates at nucleotide 2069 and terminates at nucleotide 2399, and it encodes a protein of 110 amino acids. ORF-C is a large ORF (366 amino acids)

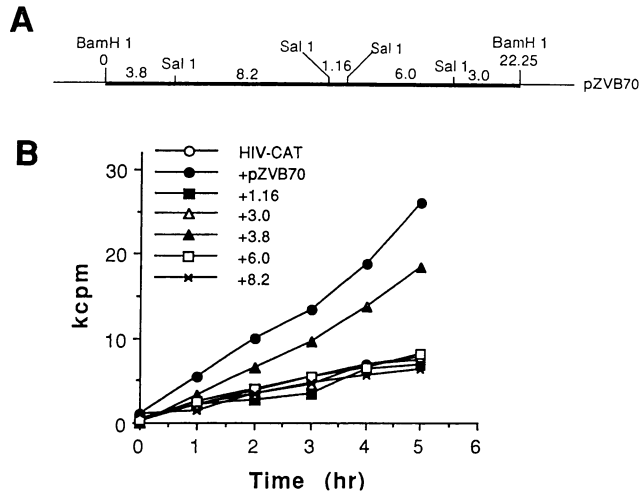


FIG. 4. (A) Schematic diagram showing the *SalI* restriction sites which mapped to the pZVB70 fragment. (B) CAT activity in CV-1 cells that were cotransfected with HIV-CAT and different *SalI*-cut subfragments of pZVB70. pZVB70 represents the intact plasmid, and the numbers indicate the size in kilobases of each pZVB70 DNA subfragment that was cotransfected with HIV-CAT.

located on the opposite DNA strand. Its amino terminus is located outside the 3.8-kb fragment. Therefore, it is unlikely that ORF-C encodes a functional protein that is involved in *trans* activation observed with the 3.8-kb fragment.

ORF-A is responsible for *trans* activation of the HIV LTR. To determine which ORF within the *SalI* 3.8-kb fragment is responsible for *trans*-activation activities, the 3.8-kb fragment was further characterized by restriction enzyme digestion and tested for HIV-CAT *trans* activation. One of the enzymes tested, *AccI*, eliminated ORF-B but not ORF-A, and the *AccI*-digested 3.8-kb fragment still retained *trans*-activation ability (data not shown). Thus, ORF-A may be responsible for the *trans* activation of the HIV LTR. To further confirm these results, both the 3.8-kb fragment and the 1.86-kb fragment containing ORF-A were subcloned and designated p3.8 and p1.86, respectively (Fig. 6). In addition, the ORF-A coding sequence was amplified by using the polymerase chain reaction, subcloned downstream of the Rous sarcoma virus (RSV) LTR, and designated pB701 (Fig. 6). These subclones, p3.8, p1.86, and pB701, were cotransfected along with HIV-CAT into both CV-1 cells and human T cells (HSB-2) and tested for *trans*-activation activities. All plasmid constructs containing ORF-A were found to be

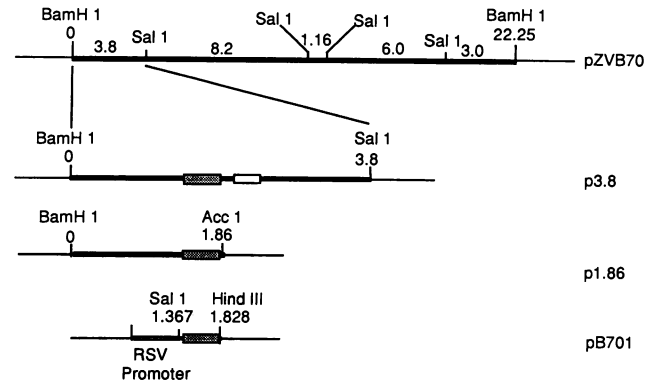


FIG. 6. Schematic diagram showing the map of plasmid pZVB70 and its various subclones. The map of pZVB70 indicates where the *SalI*-cut sites are located. p3.8, p1.86, and pB701 represents the subclones of pZVB70. Both p3.8 and p1.86 were subcloned into pUC plasmids. pB701 was subcloned into a plasmid containing the Rous sarcoma virus (RSV) promoter. The stippled boxes represent ORF-A, and the open box represents ORF-B.

active in both CV-1 cells and T cells (Fig. 7), indicating that ORF-A may indeed be the primary gene responsible for activation of the HIV promoter. It is of interest to note that in CV-1 cells (Fig. 7A), pB701, which uses Rous sarcoma virus LTR as a promoter, possesses lower *trans*-activating activity than does p1.86, which uses the endogenous HHV-6 promoter. However, this difference was not significant in HSB-2 cells. In HSB-2 cells, the cloned plasmids always gave slightly lower *trans*-activation activities than did the intact pZVB70 (Fig. 7B). It is possible that a second gene in pZVB70 also participates in the *trans* activation of the HIV LTR in HSB-2 cells. Nevertheless, these results clearly demonstrate that ORF-A is one of the major HHV-6 gene products that *trans* activates the HIV LTR in both CV-1 and human T cells. This ORF was designated B701. Our sequence analyses show that B701 encodes a protein of 143 amino acids (Fig. 8). Two potential promoters with TATAA-like sequence are found at positions 5' of the first ATG, and a potential polyadenylation site is found at the 3' end (Fig. 8).

***trans* activation of HIV LTR by B701 also involves the NF- κ B region.** To further confirm that B701 functions in a similar fashion to the HHV-6 and pZVB70 fragments in *trans* activation of the HIV LTR, pB701 was cotransfected along with either the intact HIV promoter or the mutant NF- κ B promoter in both CV-1 (Fig. 9A) and HSB-2 (Fig. 9B) cells.

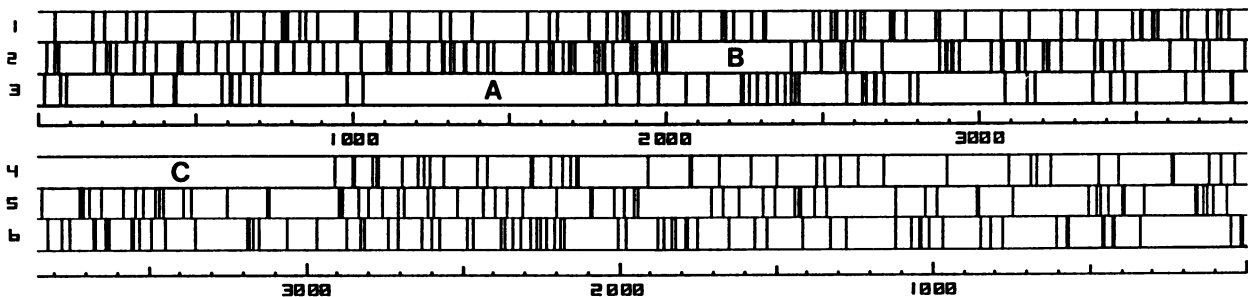


FIG. 5. Analysis for potential ORFs encoded by the 3.8-kb fragment of pZVB70. The three identified ORFs larger than 100 amino acids are designated A, B, and C. 1, 2, and 3 represent reading frames in the first DNA strand; 4, 5, and 6 represent reading frames in the opposite strand.

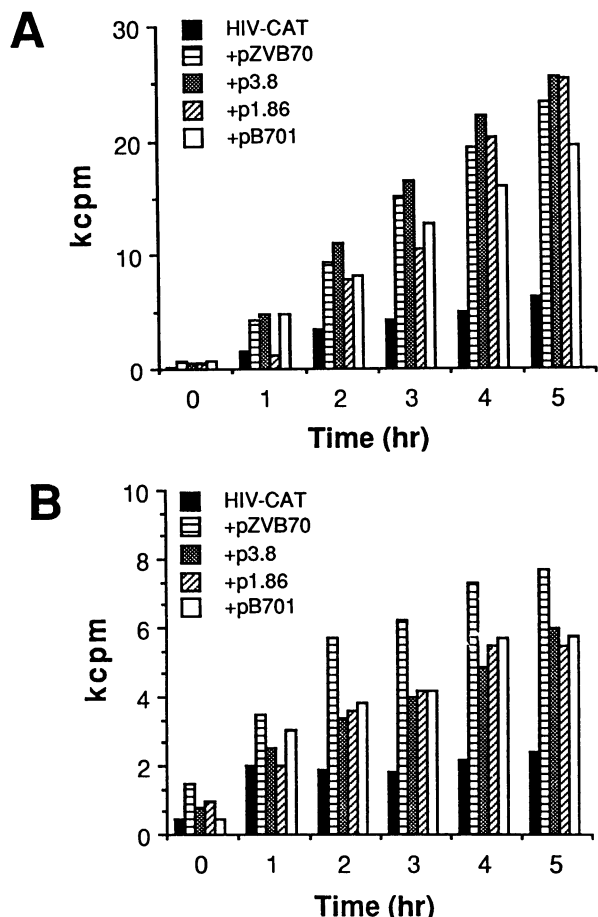


FIG. 7. CAT assays of cells that were transfected with HIV-CAT alone or cotransfected with various subclones of pZVB70 (Fig. 6). (A) Transfection studies done in CV-1 cells; (B) studies done in T cells.

As expected, *trans* activation was abolished when the NF- κ B mutant promoter was used. Furthermore, *trans* activation of the intact HIV promoter in HSB-2 by pB701 is higher than the *trans* activation obtained with HHV-6 infection (Fig. 9B). Together, these results suggest that the B701 gene of HHV-6 encodes a protein which may be responsible for most of the *trans*-activating activity on the HIV promoter by HHV-6.

DISCUSSION

Activation of the HIV LTR by exogenous viral infections, such as by herpesviruses, is apparently a complex process. For HSV-1, at least two different immediate-early gene products, ICP0 and ICP4, have been shown to enhance the expression of HIV LTR-CAT constructs (1). However, these stimulatory effects on the HIV LTR by cloned genomic HSV-1, ICP0, and ICP4 fragments varied with cell type (26), indicating that the cellular environment plays an important role in *trans* activation. Our study shows that *trans* activation of the HIV-LTR by the HHV-6 gene fragment B701 in a nonlymphoid cell line (CV-1) was as effective as that observed in a human T-cell line (HSB-2). It is likely that these two cell lines involve similar cellular factors, such as NF- κ B, in mediating the *trans* activation. Studies using a larger panel

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GACATTCGCTTTTAAACGAAAATTGATGATGTTCTTCAATCGTTGATGATGCGCAGGAT
1378
AAATTTAGGAAATTCGCTACTATCGTTTAAATAAGTGGGAGGCATTACATTTATGAAG
M K
2
TCITGCTTGGAAAATAACGATGGAGTATTGTTTTATTCTTACTAGGAAAAGAGATTTT
S C L E N N D G V F V L F L T R K E D F
CCTAGTAGGATGGAATGGAATTTCTTTTCGGATGCTACGAGAGATGCGCGTATCACGGG
P S R M E W N F F S D V Y E S M P Y H G
CAGGTATCGGAAAGTATTGGAAAAACATTACTGTATCCTAAAACGATGTTTGTGATGATG
Q V I G S I G K T L L Y P K T M F V M M
GATTTATCGGGGCAATTTATGGTATTGATACGATCGGAAACGGAAATGGATCGTGTGT
D L S G A I Y G I D T I G T G I G S C V
AAAATAGCAGACGATTTTGAATCTTTTTAAGACAAGGGATGTTAGGGGATATCGTCGC
K I A D D F E S F L R Q G I V R G Y R R
TATAAGTTTTCCATAGGAACATTAGCACCGTCCAAGAAATTTGCCGTTGTGTCCCCAT
Y K F F H R N I S T V Q E I L P L C P H
ACTTCTAGGGCCACATTCTCGAATAATTAACAATTATGATTTATCATCTGAGGATGAC
T S L G P T F S N N Y N Y D L S S E D D
1805
GAGTAAATAGATACGTCATACCTTGGAGTTGATTTAACTTGATGATGAGAGATGTTAGTA
E #
142
TACAGAAATAAATTTTAGCCGATGATAATATTGTCGTTGTAGTTTTAAATCTCGTTT
    
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FIG. 8. Nucleotide sequence and potential amino acid sequence of ORF-A (B701). Numbers above the nucleotide sequence represent nucleotide numbers; those at the right represent amino acid numbers. The two potential TATA boxes found at the 5' end of the coding region, the termination codon TAA, and the potential polyadenylated signal sequences are highlighted by boldface.

of different cell types need to be carried out to further confirm these observations.

Our transfection studies show that cloned B701 fragment activates the HIV LTR as effectively as does the entire 22.25-kb pZVB70 fragment and that this activation is at least as effective as, if not better than, activation by HHV-6 infection. These data suggest that B701 may indeed be the primary HHV-6 gene responsible for activation of HIV. Previous studies by Ensoli et al. (7) and us (13, 14) have demonstrated that activation of HIV by HHV-6 involves the

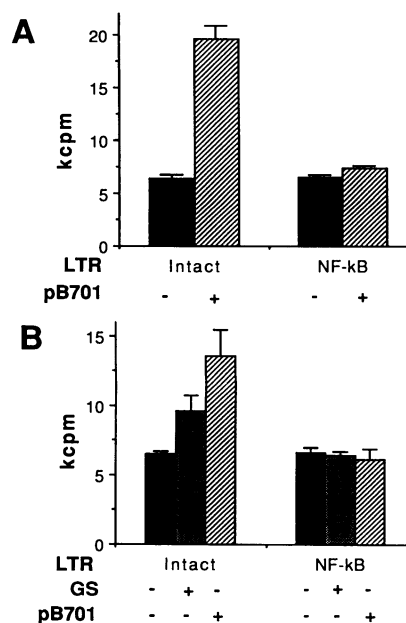


FIG. 9. Transfection studies done in CV-1 cells (A) or T cells (B) with either intact HIV-CAT or mutant NF- κ B CAT. Cells were transfected with HIV-CAT alone, cotransfected with HIV-CAT and pB701, or infected with HHV-6 (GS). Shown are counts obtained at 5 h after initiation of the assay.

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