Journal of NeuroVirology (1998) 4, 495–511

http://www.jneurovirol.com

Cytomegalovirus and human herpesvirus-6 *trans*-activate the HIV-1 long terminal repeat via multiple response regions in human fetal astrocytes

Micheline McCarthy[1,2,3], Denise Auger[1,2], Jun He[4] and Charles Wood[4]

[1]Department of Veterans Affairs Medical Center, Miami, Florida 33125, and Departments of [2]Neurology and [3]Microbiology and Immunology, University of Miami School of Medicine, Miami, Florida 33101; [4]School of Biological Sciences, University of Nebraska, Lincoln, Nebraska 68588, USA

Cytomegalovirus (CMV) and human herpesvirus-6 (HHV-6) infection stimulated HIV-1 replication and *trans*-activated the HIV-1 promoter (the long terminal repeat or LTR) to a similar extent in transfected, nonimmortalized, human fetal astrocytes. CMV infection increased basal LTR expression by approximately sevenfold, while HHV-6 infection increased basal LTR expression by fourfold. This enhancing effect required cell-cell contact between CMV-infected or HHV-6-infected and LTR-containing cells. To determine the target regions on the HIV promoter that respond to CMV and HHV-6 *trans*-activation, several modified LTR-reporter gene constructs were tested. Loss of functional NFκB, Sp1, or upstream modulatory sites on the LTR caused significant reduction of basal LTR expression in astrocytes. These elements also mediated the *trans*-activation events during HHV-6 or CMV infection in astrocytes, though to varying degrees. Electrophoretic mobility shift assays (EMSA) indicated that core, enhancer, and upstream modulatory regions of the LTR interacted specifically with nuclear proteins from both uninfected and CMV- or HHV-6-infected human fetal astrocytes. CMV or HHV-6 infection did not appear to induce unique, LTR-specific nuclear binding proteins, but rather enhanced the relative proportion of some of the existing protein complexes, in particular, the complexes formed with the AP-1 binding sites on the HIV-1 LTR (nt −354 to −316). Our data suggest that CMV or HHV-6 *trans*-activation of HIV LTR activity in human fetal astrocytes proceeds via intracellular molecular interactions involving herpesviral gene products, cellular proteins, and multiple sites on the LTR upstream of the TATA box. The pattern of LTR activity in astrocytes suggests that host cell factors modulating HIV expression may differ from those dominant in T-cells or immortalized astroglia, and this could contribute to differences in the astrocyte's ability to support HIV replication.

Keywords: Human immunodeficiency virus; astrocytes; AIDS; herpesvirus infections; cytomegalovirus; human herpesvirus-6

Introduction

Neurological disease associated with Human Immunodeficiency Virus type 1 (HIV-1) infection presents a fundamental paradox: global depression of cognitive and motor function despite relatively scarce direct HIV-1 infection of neural cells (Spencer and Price, 1992). This paradox invokes amplification mechanisms and/or co-factors to explain the connection between HIV-1 infection and clinical disease. Thus, the neuropathogenesis of HIV-1 infection is increasingly viewed as a progressive cascade of events initiated by HIV-1 gene products, amplified by immune factors and glial cells, with ultimately toxic effects on neurons (Blumberg *et al*, 1994; Lipton and Gendelman, 1995). Productive HIV-1 infection occurs in monocytes, including blood-derived macrophages and brain resident microglia, which release viral-encoded products and soluble inflammatory substances (Lipton and Gendelman, 1995). Latent or restricted HIV-1 infection occurs in astrocytes, which may function as a virus reservoir (Blumberg

Correspondence: M McCarthy
Received 21 January 1998; revised 28 April 1998; accepted 14 May 1998

et al, 1994) and harbor an abundance of viral regulatory transcripts (Tornatore *et al*, 1994b). Two recent studies have documented postmortem evidence for HIV-1 infection in as much as 20% of subcortical astrocytes in postmortem pediatric AIDS brains (Saito *et al*, 1994; Tornatore *et al*, 1994a). HIV-1 DNA sequences have also been identified in cortical and subcortical astrocytes in adult AIDS cases (Johnson *et al*, 1996). The relatively limited ability of HIV to replicate in astrocytes could be due to a post entry block in the expression or function of cellular factors necessary to support HIV structural gene expression. There is, then, a potential to increase the viral quantity or 'load' in the central nervous system (CNS) when cytokines or other factors that can overcome this block reactivate HIV-1 gene expression (Blumberg *et al*, 1994). Factors that affect cell proliferation or activation of transcription, such as cytokines (Tornatore *et al*, 1991), DNA virus gene products, or superinfection by Herpesviruses (Koval *et al*, 1995; Ho *et al*, 1991; Duclos *et al*, 1989), have been shown to stimulate HIV gene expression in astroglial cells *in vitro*.

The study of *trans*-activation by, or molecular interactions between, human herpesviruses and HIV-1 has focused attention on the role these viruses may play as co-factors during the course of HIV-1 infection (Laurence, 1990). Of the human herpesviruses, cytomegalovirus (CMV) and human herpesvirus-6 (HHV-6) have similar molecular properties (Lawrence *et al*, 1990) and are capable of replicating or persisting in CD4+ lymphocytes (Sissons *et al*, 1986; Takahashi *et al*, 1989) as well as invading the CNS. These properties make them particularly well-suited to function as co-factors in HIV-1 neuropathogenesis. There is a growing body of evidence documenting active CMV or HHV-6 infection within the CNS of HIV-infected patients. CMV is probably the most common opportunistic CNS virus in patients with HIV-1 infection or AIDs (reviewed in Harrison and McArthur, 1995), and can cause a subacute encephalopathy which follows an acute to sub-acute course with rapid cognitive decline (Fiala *et al*, 1991). Double-immunolabeling *in situ* techniques detecting viral nucleic acid indicate that 12% of cells infected by CMV are also infected by HIV-1 in subcortical regions of the brain (Nelson *et al*, 1988). HHV-6 has been isolated from patients in various stages of HIV-1 infection (reviewed in Lusso and Gallo, 1995), and postmortem studies have demonstrated active and disseminated HHV-6 infection in AIDS patients (Lusso and Gallo, 1995; Saito *et al*, 1995). Dual HHV-6 and HIV-1 infection of brain has now been documented in both pediatric (Saito *et al*, 1995) and adult (Achim *et al*, 1994) AIDS, with HHV-6 occurring in oligodendrocytes, astrocytes, microglia, and neurons.

The regulatory sites on the HIV-1 promoter or long terminal repeat (LTR) are the likely loci of molecular interactions between Herpesviruses and HIV-1 (Gendelman *et al*, 1986; Laurence, 1990) whenever coinfection of single cells with HIV-1 and a specific Herpesvirus occurs. The regulatory sites are functionally arranged into a proximal core region which binds the Sp1 transcription factors, an enhancer element with NFκB binding sites, and an upstream modulatory region that variably binds numerous transcription factors including ATF/CREB (Krebs *et al*, 1997) or AP-1 (Canonne-Hergaux *et al*, 1995). *In vitro* CMV will *trans*-activate or increase mRNA and protein synthesis directed by the HIV-1 LTR (reviewed in Spector *et al*, 1994). The *trans*-activating effect of CMV infection can be reconstituted at least in part with products of the CMV immediate early (IE) gene regions in both human fibroblasts (Barry *et al*, 1990; Biegalke and Geballe, 1991; Ghazal *et al*, 1991; Walker *et al*, 1992) as well as primary human astrocytes (Ho *et al*, 1991), although *trans*-activation varies considerably among different cell types (Barry *et al*, 1991). Like CMV, HHV-6 has been reported to stimulate HIV production in dually infected T-lymphocytes, causing rapid cell death (Lusso *et al*, 1989). At the molecular level, early HHV-6 gene products *trans*-activated HIV LTR-directed gene expression in T-cells, probably acting via the cellular transcription factor NFκB (Horvat *et al*, 1991; Geng *et al*, 1992; Zhou *et al*, 1994). The Sp1 binding sites on the LTR may be responsive to an HHV-6 IE gene product (Martin *et al*, 1991).

We have previously developed nonimmortalized, highly enriched human fetal astrocyte cultures in defined medium and demonstrated that these cultures support transient expression of HIV-1 after infection (McCarthy *et al*, 1998), and transient expression by transfected recombinant plasmids containing the HIV LTR linked to either the chloramphenicol acetyltransferase (CAT) or β-galactosidase (β-gal) reporter genes (McCarthy *et al*, 1995). These cells will also support productive infection by CMV (McCarthy *et al*, 1995) or HHV-6 (He *et al*, 1996). Thus the HIV LTR-CAT-transfected astrocyte cultures are a useful system with which to study how CMV or HHV-6 infection influences HIV replication and HIV LTR-directed gene expression in brain glia. In this study we have demonstrated that CMV infection or HHV-6 infection summarily *trans*-activate the HIV LTR or enhance HIV-1 replication to a similar extent in nonimmortalized human fetal astrocytes. Multiple responsive elements on the HIV LTR appear to mediate the *trans*-activation events during HHV-6 or CMV infection in these cells.

Results

CMV and HHV-6 stimulate p24 production by HIV-infected human fetal astrocytes

CMV or HHV-6 infection could potentially be a co-factor in enhancing HIV-1 replication in neural cells

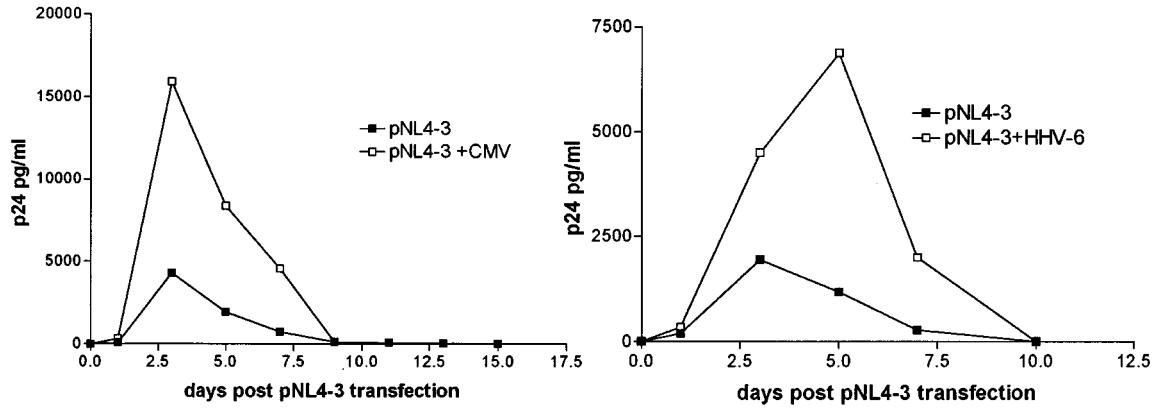


Figure 1 Concurrent infection by CMV (left panel) or HHV-6 (right panel) enhances soluble p24 antigen production by pNL4-3-transfected human fetal astrocytes. Subconfluent cells were mock-or infected with CMV(AD169) or HHV-6(GS) and transfected with pNL4-3 via cationic liposomes as described in Materials and methods. Culture supernatants were collected at transfection (time 0) and every 48 h with change of medium for quantitative p24 assay.

such as astrocytes. CMV infection has been reported to enhance p24 production by proviral DNA-transfected human fetal astrocytes (Ho *et al*, 1991; McCarthy *et al*, 1998). To demonstrate that similar enhanced HIV-1 replication occurs with HHV-6, CMV- or HHV-6-infected astrocytes were transfected with proviral DNA from the lymphotropic strain NL4-3, and soluble p24 antigen synthesis was then assayed (Figure 1). Concurrent HHV-6 or CMV infection similarly stimulated approximately a threefold increase in the peak p24 values during the productive phase of the HIV-1 infection in astrocytes lasting from days 2–8 post transfection. CMV or HHV-6 infection also tended to broaden the temporal profile of p24 production. However, neither CMV nor HHV-6 stimulated a persistent infection of astrocytes, as measured by p24 production.

CMV infection or HHV-6 infection can trans-activate the HIV LTR in human astrocytes

Since CMV or HHV-6 infection activated HIV-1 replication in astrocytes, it is important to determine whether such activation could occur via the stimulation of HIV LTR expression. Indeed, concurrent infection by CMV or HHV-6 significantly increased transient HIV LTR-directed CAT reporter gene expression in human fetal astrocytes (Figure 2). Concurrent CMV infection increased basal LTR-CAT expression by approximately sevenfold, while HHV-6 infection increased basal LTR-CAT expression by approximately fourfold. Within the error of the mean CAT expression values depicted in Figure 2, there is not a significant difference between the *trans*-activating effects of CMV infection (6.8-fold activation) and HHV-6 infection (4.2-fold activation) on HIV LTR expression in human fetal astrocytes, though both viruses significantly activate the LTR-CAT above the respective basal (control) values. For comparison, the effect of tat alone on HIV LTR-CAT expression in parallel mock-infected human fetal astrocytes was also assayed. HIV LTR activation by tat alone ranged from 2–5-fold, with a mean of approximately fourfold. In general the *trans*-activating effect of tat was similar to that of either CMV infection or HHV-6 infection. This tat effect in non-immortalized human fetal astrocytes is modest when compared to the several hundred fold activation by tat observed in T-lymphocytes (Sodroski *et al*, 1985; Horvat *et al*, 1991), and this likely reflects cell type-specific influences on LTR expression. CMV or HHV-6 infection prior to or after LTR-CAT transfection (see Methods) activated basal LTR-CAT expression to a similar extent (data not shown). This confirmed that CMV or HHV-6 infection did not nonspecifically increase plasmid DNA uptake or transport.

Because CMV and HHV-6 infected human fetal astrocytes with such high efficiency, CMV- or HHV-6-mediated *trans*-activation of HIV-1 p24 or LTR-CAT expression occurred under conditions in which there was a high probability that about 2–3% of the cells contained both transfected HIV-1 proviral DNA or LTR-CAT DNA and replicating CMV or HHV-6. CMV antigen was present in greater than 80% of astrocytes in the culture by 48 h post transfection as confirmed by immunofluorescence assay (IFA) for viral structural antigens (McCarthy *et al*, 1995). HHV-6 antigen was present in 50–70% of cells in the culture by 48 h post transfection as confirmed by IFA for viral antigens (He *et al*, 1996). The percent of successfully transfected cells was 2–3% (see Materials and methods). Thus the pervasive presence of CMV or HHV-6 in these experimental conditions suggested that *trans*-activation proceeded via intracellular interactions between herpesvirus gene products or herpes-

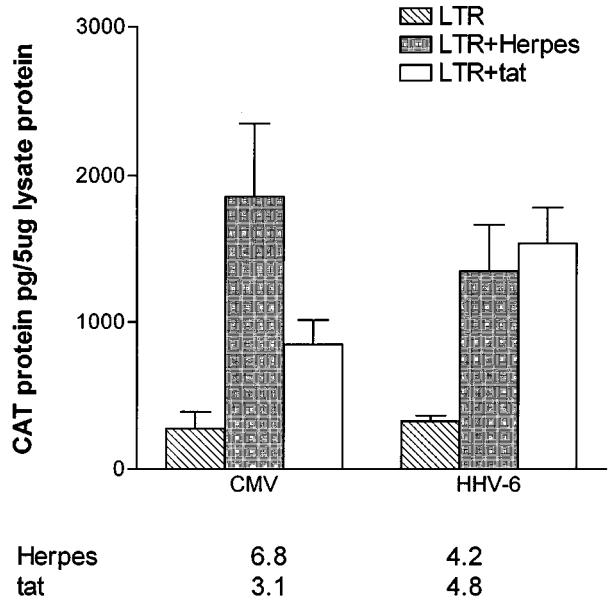


Figure 2 HIV LTR-CAT expression in human fetal astrocyte cultures, determined as CAT protein per 5 μg cell lysate protein $\pm$ standard error. Astrocytes (10^6 cells) transfected with 2 μg LTR-CAT plasmid DNA were concurrently infected by HHV-6 or CMV (both viruses designated as 'Herpes' in bar graph legend), or mock-infected and co-transfected with 4 μg RSV-tat plasmid to determine the effect of tat on LTR activity. Numbers in the table below each bar graph represent the fold activation of the LTR-CAT construct by HHV-6 or CMV infection or by co-transfected tat, normalized to basal CAT expression by intact LTR-CAT. The data are derived from independent experiments using cultures derived from different tissue specimens. The CMV data were derived from 9 experiments, while the HHV-6 data were derived from 12 experiments.

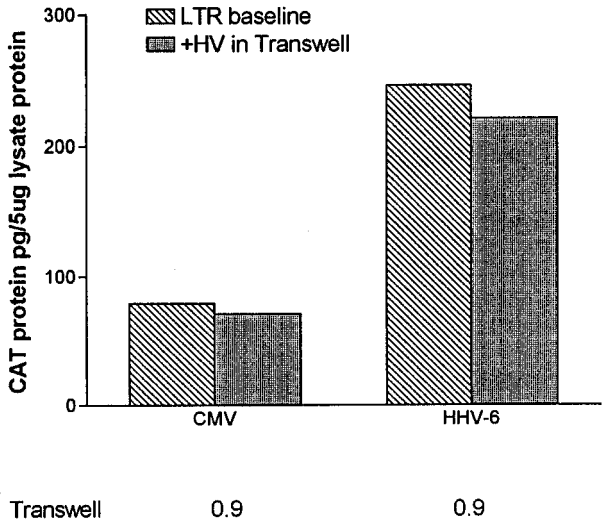


Figure 3 Activation of HIV LTR-CAT expression requires cell-cell contact betwen HHV-6- or CMV-infected and LTR-CAT-transfected astrocytes. CMV-infected or HHV-6-infected astrocytes were seeded onto Transwell inserts (CoStar, Cambridge, MA) and incubated above LTR-transfected astrocytes seeded in the well bottoms of 6-well tissue culture plates ('+HV in Transwell'). In parallel, mock-infected astrocytes were seeded onto Transwell inserts, and incubated above LTR-transfected astrocytes seeded in the well bottoms ('LTR baseline'). The table below the bar graph presents the HHV-6- or CMV-associated fold activation of LTR-CAT expression in Transwell culture, i.e. LTR-CAT activity in Transwell divided by LTR-CAT baseline.

induced cellular products and the HIV LTR. Additional experiments did show that soluble factors such as cytokines could not account for the increased LTR activity in LTR-CAT-transfected astrocytes infected by CMV or HHV-6. The enhancing effect of CMV or HHV-6 infection on HIV LTR activity in astrocytes appeared to require cell-cell contact between virus-infected and LTR-containing cells. HHV-6- or CMV-infected astrocytes were seeded on Transwell inserts (Transwell Clear, CoStar, Cambridge, MA) in 35 mm tissue culture wells to physically separate them from LTR-CAT-transfected astrocytes which were plated on the well bottoms. Under these conditions, only basal levels of HIV LTR-CAT expression were detected in these transfected astrocytes (Figure 3), and there was no effect of CMV or HHV-6 infection on the baseline HIV LTR activity.

Modulatory and enhancer regions affect basal HIV LTR activity in human astrocytes

To delineate the target regions on the HIV-1 promoter as well as the cellular factors that may participate in the promoter's interactions with CMV and HHV-6, several modified HIV LTR-CAT constructs were tested. These included constructs with mutations or deletions in the enhancer (NFκB) and core (Sp1) domains, plus additional LTR constructs truncated in the core (-29LTR, -57LTR) or modulatory (-147LTR) upstream regulatory regions (Figure 4A). Consistent with what was observed in T-cells (Horvat *et al*, 1991), unstimulated or basal CAT expression by the modified LTR-CAT constructs was significantly less than that of an equivalent amount of the intact HIV LTR-CAT transfectd into parallel astrocyte cultures (Figure 4B). Loss of functional NFκB binding sites by deletion (-57LTR, -29LTR) or mutation (mutant NFκB LTR) (Nabel and Baltimore, 1987) caused significant reduction of basal CAT expression, ranging from 1–9% of the CAT values achieved with the intact HIV LTR-CAT. Deletion of the Sp1 binding sites reduced basal CAT expression to approximately 21% of intact HIV LTR-CAT. The -147LTR promoter, which is deleted of upstream modulatory elements but contains all NFκB and Sp1 sites as well as the TATA box, was essentially inactive in human fetal astrocytes. Basal CAT expression driven by this promoter was just 2% of intact HIV LTR-CAT.

This truncated promoter drives higher than basal levels of CAT expression in primary T-lymphoblasts, presumably because the upstream modulatory elements (the 'NEG' regulatory region) function to suppress promoter activity in lymphocytes (Horvat *et al*, 1991). In human fetal astrocytes, the lack of CAT expression by this truncated promoter suggests that the upstream elements may exert a positive regulatory effect on HIV LTR activity.

Modulatory and enhancer regions can participate in Herpesvirus-mediated trans*-activation of the HIV LTR in human fetal astrocytes*

CMV infection could activate the mutant LTR-CAT constructs and compensate to a relative degree for the transcriptional deficiencies in all the mutant promoters excepting the minimal −29LTR construct. CMV infection stimulated the Sp1-deleted (dlSp1 LTR) and mutant NFκB LTR promoters very effecively to CAT expression levels approximately

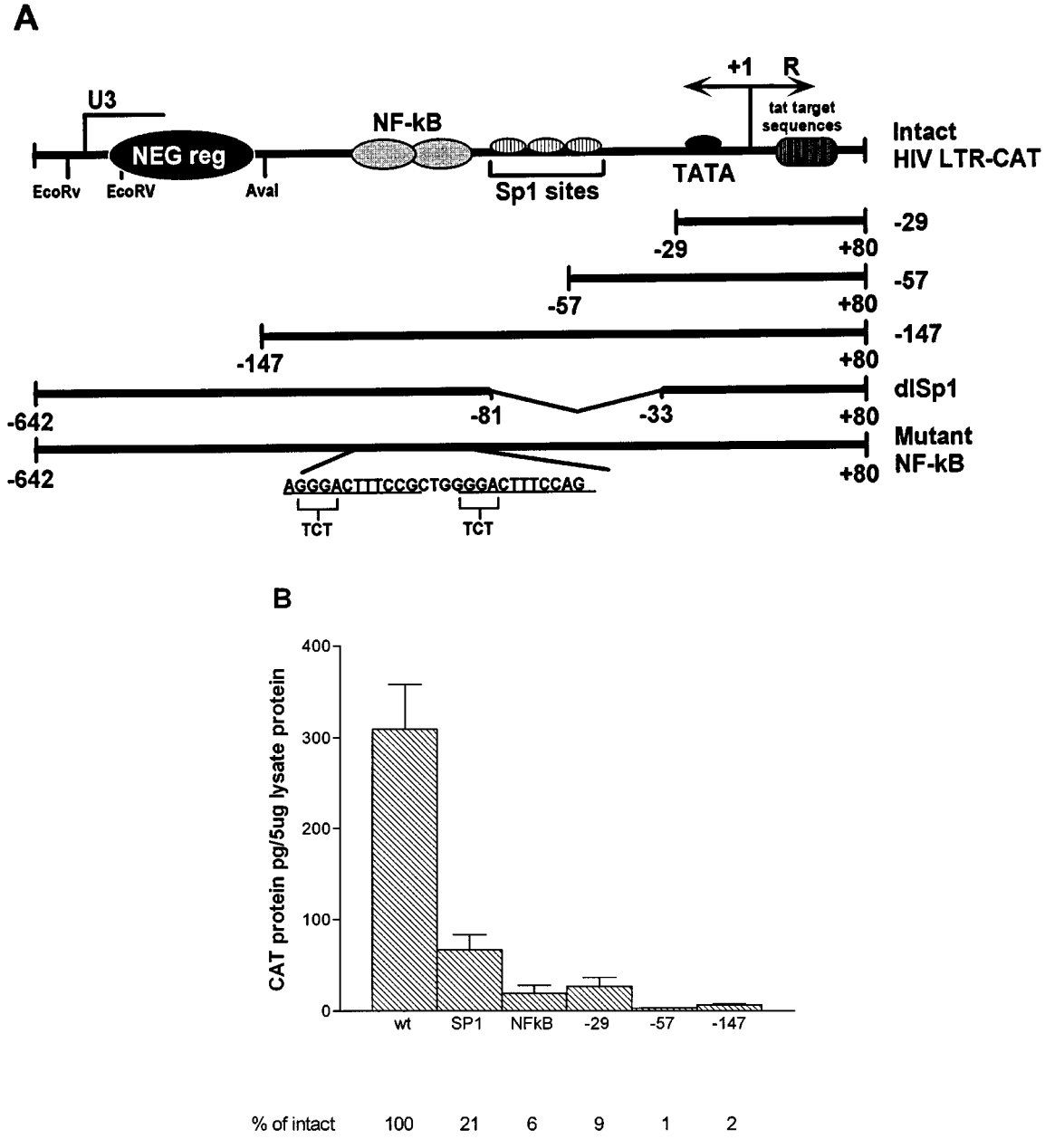
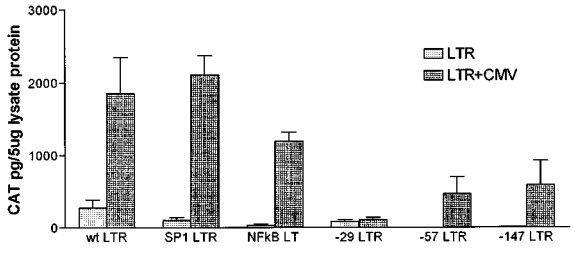


Figure 4 (**A**) Schematic diagram of the mutant HIV LTR-CAT constructs used in transfection studies in human fetal astrocyte cultures. Derivation of the constructs is described under Materials and methods. (**B**) Basal CAT expression by these constructs. The table includes the values for CAT expression by these constructs expressed as a percentage of the CAT expression by the intact LTR-CAT construct. Data in the table represent the mean of four independent assays ± standard error.

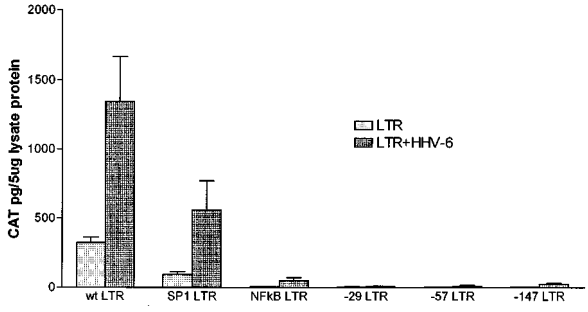
4–8-fold higher than basal intact LTR-CAT expression (Figure 5), even though the basal activities of these two mutant promoters were lower than that of the intact LTR. CMV infection stimulated CAT expression by the truncated −57LTR and −147LTR constructs to levels approximately twofold higher than basal intact LTR-CAT expression. Fold activation of the dlSp1, NFκB, −57LTR and −147LTR-CAT constructs, when referred to basal CAT expression by the homologous construct, ranged from 20.9 to 154.7, much higher than that of intact LTR-CAT (fold activation 6.8). This suggests that CMV-mediated transactivation events can occur at multiple sites, since CMV infection will activate all the mutant LTR constructs except the −29LTR. The CAT activity of the minimal −29LTR promoter was not significantly increased (fold activation 1.3) by concurrent CMV infection. Activated mutant LTR-CAT expression was normalized to unstimulated intact LTR-CAT expression ('normalized fold activation') to assess whether CMV infection could offset the functional defects in the various mutant LTR constructs (Figure 5). Normalized fold activation values for dlSp1 LTR (7.8) and NFκB LTR (4.4) were comparable to that of intact LTR-CAT (6.8). The other mutant promoters (−29LTR, −57LTR, −147LTR) had much lower CMV-activated CAT expression levels and this was reflected in lower normalized fold activation values (0.4, 1.7, 2.2, respectively). The truncated promoters −57LTR and −147LTR have deletions of the enhancer and/or upstream modulatory sequences, while the dlSp1 LTR and mutant NFκB LTR contain these upstream LTR sequences. Deletion of these upstream modulatory sequences reduced the CMV-activated CAT expression values and the normalized fold activation values to a relatively greater extent than mutations in the enhancer region, suggesting that these upstream LTR sequences participate in the positive modulating effect that CMV infection exerts on HIV LTR-driven transcription in human fetal astrocytes.

As with CMV infection, HHV-6 infection could activate all the mutant LTR constructs except the −29LTR, suggesting that HHV-6-mediated *trans*-activation events can occur at multiple sites. HHV-6 *trans*-activated the dlSp1 LTR-CAT expression by about sixfold to levels approximately twice that of the unstimulated intact LTR-CAT (normalized fold activation of 1.7) (Figure 6). This suggests that functional Sp1 sites do not solely determine HHV-6 *trans*-activation of the LTR in human fetal astrocytes. HHV-6 infection *trans*-activated the mutant NFκB LTR and the truncated promoters (−57LTR, −147LTR), but to CAT expression levels that were still much lower than those of the intact LTR-CAT (Figure 6). The mutant NFκB-LTR construct was stimulated approximately sevenfold by HHV-6 infection, but CAT levels were only 20% of the unstimulated intact LTR-CAT. The normalized fold activation values (referred to intact LTR) indicate that HHV-6 activation cannot offset functional defects from loss of upstream LTR sequences or a functional NFκB-binding element. As with CMV infection, the CAT activity of the minimal −29LTR promoter was not significantly increased (fold activation 1.3) by concurrent HHV-6 infection. Significant *trans*-activation (fold activation 3.4)



CMV (homologous)	6.8	20.9	37.4	1.3	154.7	73.4
CMV (normalized)	6.8	7.8	4.4	0.4	1.7	2.2

Figure 5 CMV-activated CAT expression by mutant HIV promoter constructs in human fetal astrocyte cultures. Astrocyte cultures (10^6 cells per dish) were mock-infected or infected with CMV prior to transfection in parallel with 2 μg of intact LTR-CAT or modified LTR-CAT plasmid DNA. The table includes the values for the CMV-mediated fold activation of each construct referred to unstimulated CAT expression by the homologous construct ('CMV(homologous)'), and the CMV-mediated fold activation of each construct referred to unstimulated CAT expression by the intact LTR ('CMV(normalized)'). Data represent the mean ± standard error of four independent assays.



HHV-6(homologous)	4.2	6.0	7.3	1.3	3.4	4.6
HHV-6 (normalized)	4.2	1.7	0.2	0.02	0.03	0.08

Figure 6 HHV-6-activated CAT expression by mutant HIV promoter constructs in human fetal astrocyte cultures. Astrocyte cultures (10^6 cells per dish) were mock-infected or infected with HHV-6 prior to transfection in parallel with 2 μg of intact LTR-CAT or modified LTR-CAT plasmid DNA. The table includes the values for the HHV-6-mediated fold activation of each construct referred to unstimulated CAT expression by the homologous construct ('HHV-6(homologous)'), and the HHV-6-mediated fold activation of each construct referred to unstimulated CAT expression by the intact LTR ('HHV-6(normalized)'). Data represent the mean ± standard error of four independent assays.

occurred with the −57LTR-CAT construct, which contains approximately 30 bp more than the minimal −29LTR. Thus, as is the case with CMV infection, LTR *trans*-activation mediated by HHV-6 infection required the LTR to have a minimum length of regulatory sequence upstream of the TATA box.

CMV and HHV-6 infection stimulate nuclear protein binding to multiple regions of the HIV-1 LTR in human astrocytes

Analysis of the data obtained from the intact and mutant LTR-CAT constructs suggests that, in human fetal astrocytes, CMV or HHV-6 infection can *trans*-activate the LTR at multiple independent sites provided a minimal length of regulatory sequence upstream of the TATA box is present within the LTR. EMSA was performed to determine whether LTR regions that participated in CMV- or HHV-6-mediated *trans*-activation in astrocytes also represent targets for binding by nuclear factors. Three oligonucleotide probes were used (Figure 7). Probe I (nt −158 to +5) contained the core and enhancer regions of the LTR, regions which are partially or entirely deleted in the −29LTR, −57LTR and dlSp1 LTR constructs. Probe II (nt −340 to −159) contained the upstream modulatory region of the LTR including three AP-1-like binding motifs (nt −333 to −327; nt −299 to −293; nt −240 to −234; Figure 7), which are deleted in the −147LTR construct. Probe SN (nt −356 to −316) overlapped the 5′ end of probe II, and contained the two 'distal' or upstream AP-1 binding motifs (nt −343 to −349; nt −333 to −327; Figure 7). The LTR sequence encompassed by probe SN has been implicated as a site of LTR activation via direct interactions with proteins of the nuclear receptor family (Canonne-Hergaux *et al*, 1995; Sawaya *et al*, 1996). Nuclear proteins extracted from mock-infected human fetal astrocytes formed specific DNA-protein complexes with all three probes, as did equivalent amounts of nuclear proteins extracted from the corresponding CMV- or HHV-6-infected cultures.

EMSA using radiolabeled probe I generated multiple DNA-protein complexes with nuclear extracts from mock-infected, HHV-6-infected, or CMV-infected astrocytes (Figure 8A). EMSA patterns differed somewhat according to whether the mock-infected astrocytes were initially inoculated with DMEM-2% FBS (Figure 8A, lane 4) to control for CMV infection, or co-cultivated with uninfected HSB2 cells (Figure 8A, lane 2) to control for HHV-6 infection. This suggests that mock infection conditions induced some nuclear protein expression in the astrocytes. We did detect low levels of TNF-α (1−10 pg/ml) in culture fluids from uninfected HSB2 cells but not in untreated astrocyte cultures (data not shown). This or similarly low levels of other cytokines could enhance nuclear protein expression in the astrocytes co-cultivated with HSB2 cells as controls for HHV-6 infection. Three DNA-protein complexes co-migrating in all EMSA patterns were noted. These were designated A1, A2 and A3 in order of increasing mobility. A3 usually migrated as a doublet. Additional DNA-protein complexes slower in mobility than A1 were inconsistently observed in EMSA patterns, and appeared to be

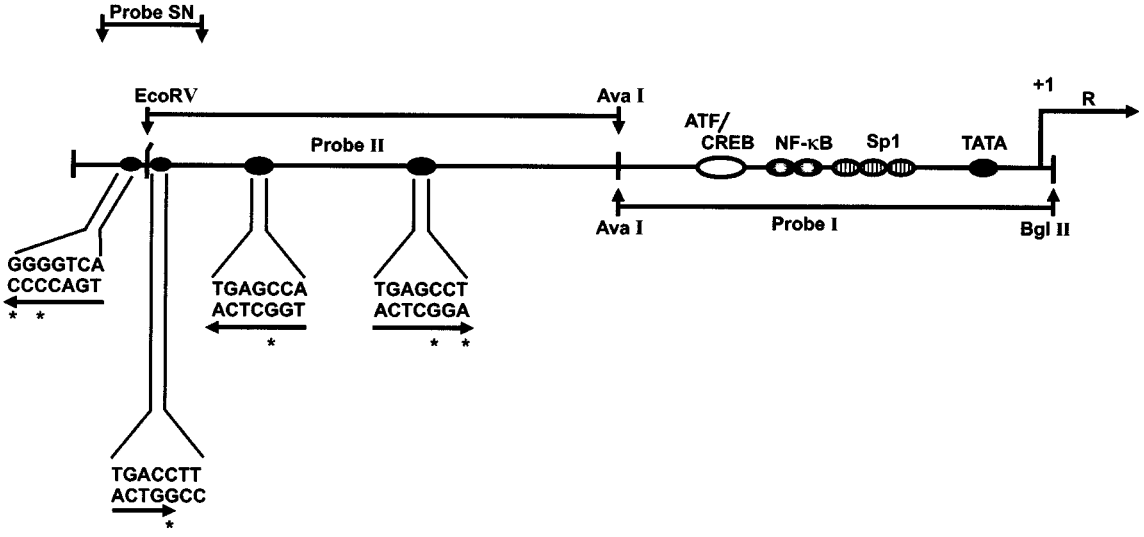


Figure 7 Schematic diagram of location of gel shift probes and putative AP-1 binding sites within HIV-1 strain NL4-3 LTR. Arrows underline the TGACTCA consensus AP-1-binding sequence, with asterisks (*) indicating nucleotide substitutions in the viral LTR sequence in that location.

due to non-specific DNA-protein binding. Nuclear extracts from HHV-6- or CMV-infected astrocytes (Figure 8A lanes 3 and 5 respectively) generated essentially the same mobility-shifted pattern as their respective mock-infected controls (Figure 8A lanes 2 and 4), but the A3 complexes from the infected cells were relatively more intense compard to the A1 and A2 complexes. Thus CMV or HHV-6 infection appeared to increase the relative intensities of these LTR-binding proteins in the A3 complex rather than induce new species of DNA-binding proteins specific for the core/enhancer region of this LTR. In competitive binding EMSA using a 50-fold molar excess of unlabeled probe I added to labeled probe and nuclear proteins, the radiolabeled A1, A2 and A3 DNA-protein complexes were competed, thereby confirming the specificity of the complexes (data not shown). Formation of complex A3 was competitively inhibited by 50-fold molar excess of unlabeled oligonucleotide containing a consensus Sp1 binding sequence (Figure 8B lane 3), while complex A1 was competed by excess unlabeled oligonucleotide containing a consensus NFκB binding sequence (Figure 8B lane 4). Thus both CMV and HHV-6 infection appeared to increase the relative intensities of astrocyte nuclear protein complexes that have Sp1-type binding activity.

EMSA using radiolabeled probe II gave rise to four major DNA-protein complexes designated B1 through B4 in order of increasing mobility (Figure 9A). With probe II, again EMSA using extracts from HHV-6- or CMV-infected astrocytes (Figure 9A lanes 3 and 5 respectively) or their respective mock-infected controls (Figure 9A lanes 2 and 4) produced the same mobility-shifted pattern. There was not much difference observed in the pattern of signal intensities from complexes formed with extracts from the HHV-6- or CMV-infected cells (Figure 9A lanes 3 and 5) as compared to their respective mock-infected controls (Figure 9A lanes 2 and 4), except that signals from complexes B2 and B3 may be slightly elevated relative to B1 and B4 after HHV-6 infection (comparing Figure 9A lanes 2 and 3). In competitive binding EMSA using a 50-fold molar excess of unlabeled probe II, the four

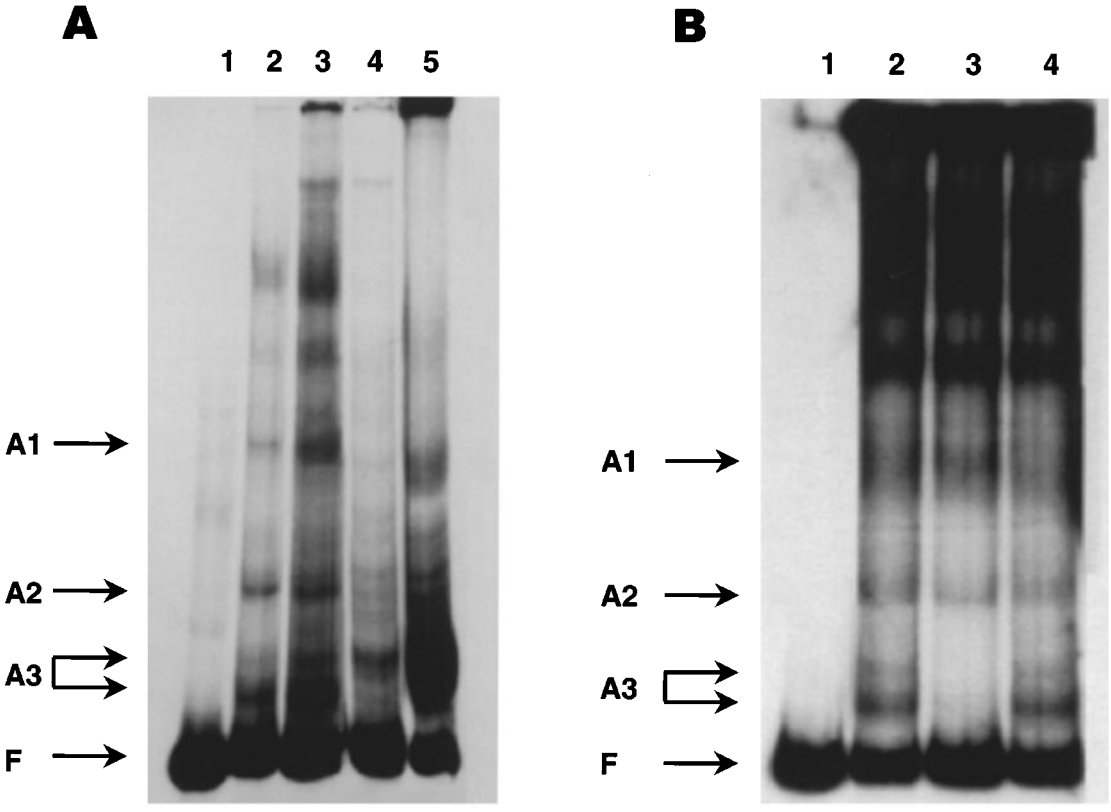


Figure 8 (**A**) EMSA using probe I (lane 1) reacted with nuclear protein extracts from mock-infected astrocytes previously incubated with uninfected HSB2 cells (lane 2), HHV-6-infected astrocytes (lane 3), mock-infected astrocytes inoculated with culture medium (lane 4), or CMV-infected astrocytes (lane 5). A1, A2, and A3 designate locations of specific DNA-protein complexes; F is free probe. (**B**) EMSA using probe I (lane 1) reacted with nuclear protein extracts from HHV-6-infected astrocytes (lanes 2–4). Specific complex formation was inhibited by a 50-fold excess of unlabeled oligonucleotide containing a consensus Sp1-binding (lane 3) or a consensus NFκB-binding (lane 4) sequence.

DNA-protein complexes had reduced signal intensity, especially B1 and B4, suggesting these were specific complexes (Figure 9B lanes 2 and 3). However, these complexes were not competitively inhibited by a 50-fold molar excess of unlabeled oligonucleotide containing the consensus AP-1 binding sequence, TGACTCA (Figure 9B lanes 2 and 4). Formation of specific DNA-astrocyte protein complexes in this region of the LTR is consistent with our LTR-CAT functional data indicating that the upstream modulatory region sequences affect LTR activity in both mock-infected and HHV-6- or CMV-infected human fetal astrocytes. The lack of competitive inhibition by the AP-1 binding sequence agrees with the findings of Canonne-Hergaux *et al* (1995) using nuclear proteins from the U373-MG human high grade astrocytoma cell line and nearly identical LTR sequences from the lymphotropic HIV-1 strain LAI.

EMSA using radiolabeled probe SN gave rise to several DNA-protein complexes (Figure 10A). In competitive binding assays using a 50-fold molar excess of unlabeled probe SN, all but the fastest migrating complexes (NS) were eliminated from EMSA patterns (Figure 10B lanes 4 and 7). The remaining five specific complexes were designated SN1 through SN5 in order of increasing mobility. The signal from complex SN1 appeared more intense relative to complexes SN4 and SN5 with nuclear extracts from both HHV-6(GS)-infected astrocytes (Figure 10A lane 5) and the corresponding control cultures that were mock-infected with HSB2 cells (Figure 10A lane 4). Complex SN1 was inconsistently detected in EMSA patterns from CMV-infected astrocytes (Figure 10A lane 3) and the corresponding control cultures (Figure 10A lane 2). The difference in mock infection conditions may have accounted for the differences between EMSA patterns derived from the two sets of mock-infected astrocytes. Complexes SN2 and SN3 gave relatively more intense signals compared to complexes SN4 and SN5 with extracts obtained from both HHV-6 and CMV-infected astrocytes (Figure 10A lanes 3 and 5) as compared to their respective mock-infected cultures. This suggests that HHV-6 or CMV infection altered the

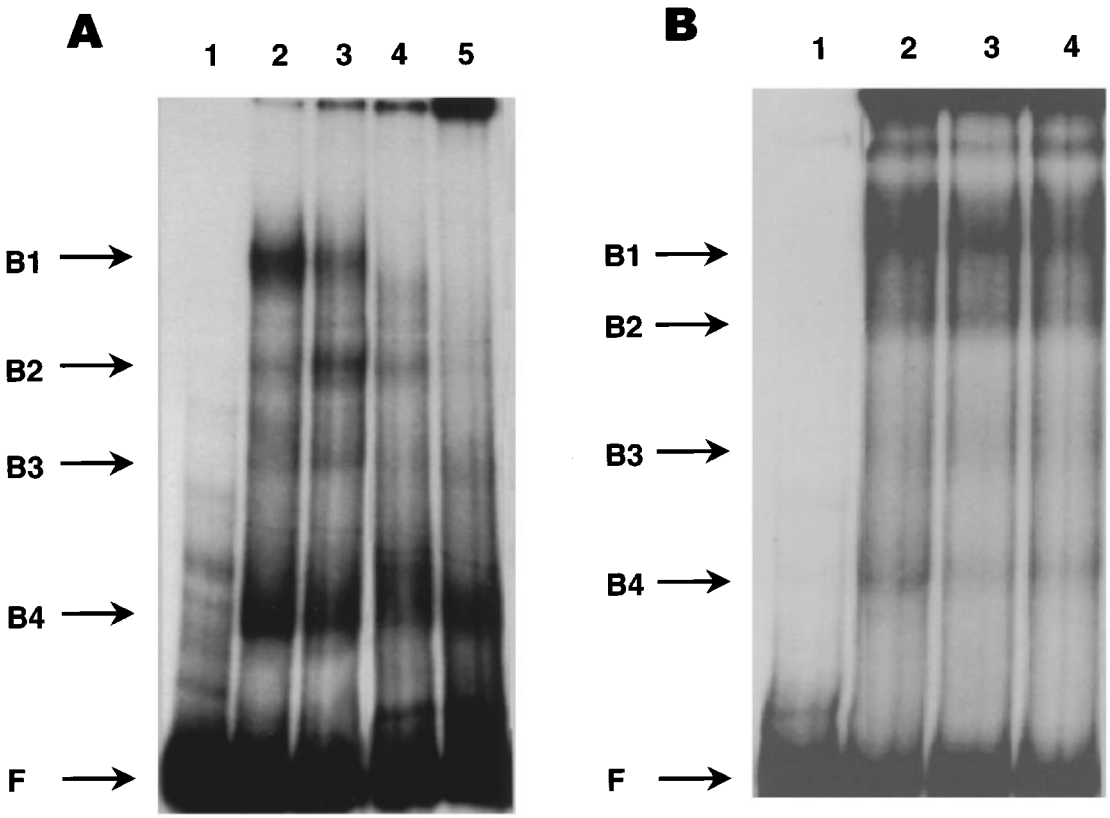


Figure 9 (**A**) EMSA using probe II (lane 1) reacted with nuclear protein extracts from mock-infected astrocytes previously incubated with uninfected HSB2 cells (lane 2), HHV-6-infected astrocytes (lane 3), mock-infected astrocytes inoculated with culture medium (lane 4), or CMV-infected astrocytes (lane 5). B1, B2, B3 and B4 designate the locations of DNA-protein complexes; F is free probe. (**B**) EMSA using probe II (lane 1) reacted with nuclear protein extracts from HHV-6-infected astrocytes (lanes 2–4), with an additional 50-fold excess of unlabeled probe II (lane 3), or with additional 50-fold excess unlabeled oligonucleotide containing a consensus AP-1 binding sequence (lane 4).

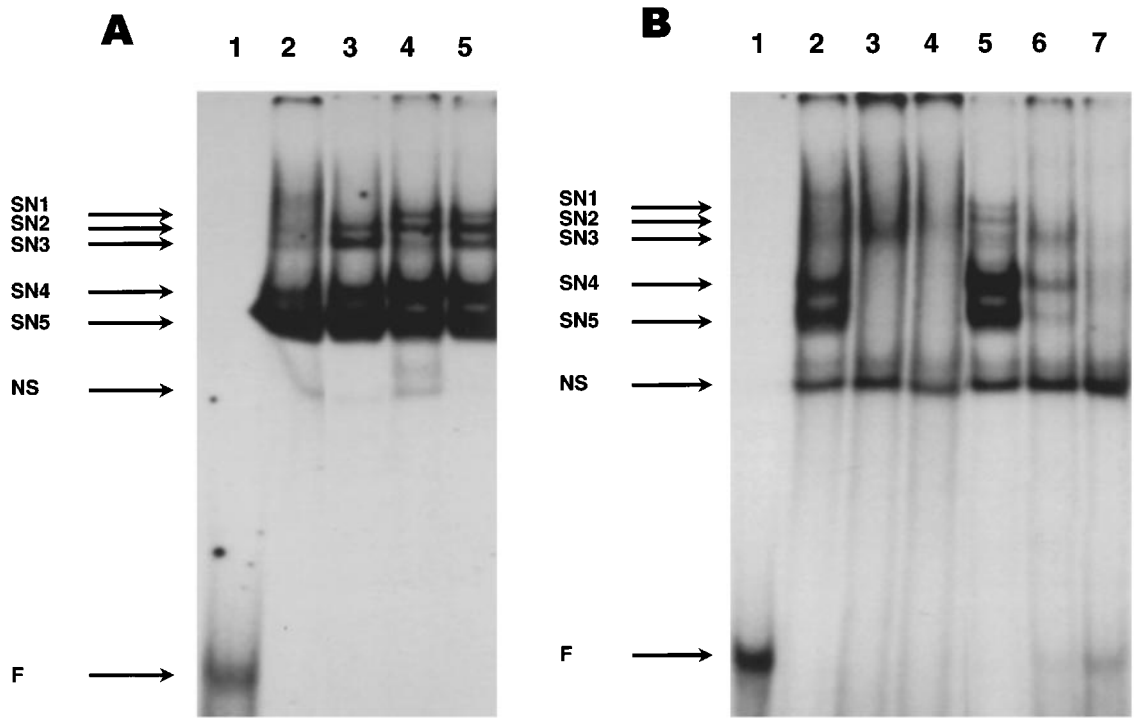


Figure 10 (**A**) EMSA using probe SN (lane 1) reacted with nuclear protein extracts from mock-infected astrocytes inoculated with culture medium (lane 2), CMV-infected astrocytes (lane 3), mock-infected astrocytes previously incubated with uninfected HSB2 cells (lane 4), or HHV-6-infected astrocytes (lane 5). SN1 through SN5 designate locations of specific DNA-protein complexes; NS are the fastest migrating non-specific complexes. F is free probe. (**B**) EMSA using probe SN (lane 1) reacted with CMV-infected astrocytes (lanes 2–4) or HHV-6-infected astrocytes (lanes 5–7). Specific complexes SN1, SN2, SN4, and SN5 were inhibited by a 50-fold molar excess of the unlabeled AP-1 binding oligonucleotide (lanes 3 and 6). All SN complexes were inhibited by a 50-fold molar excess of unlabeled probe SN (lanes 4 and 7).

proportion of nuclear proteins that complex to this region of the LTR.

Our DNA-protein complexes SN1, SN2, SN4 and SN5 were competitively inhibited by a 50-fold molar excess of the consensus AP-1 binding motif, suggesting that AP-1-like proteins do bind to this LTR region (Figure 10B lanes 3 and 6). To further investigate the specificity of these DNA-binding proteins, we performed electrophoretic gel mobility assays using antibodies directed against the Jun or Fos DNA-binding proteins to inhibit specific DNA-protein complex formation (Figure 11). The formation of complexes SN1 and SN2 was inhibited by anti-Fos or anti-Jun antibodies (Figure 11 lanes 3–5). A broadly reactive antibody against Fos proteins also suppressed formation of complex SN4 (Figure 11 lane 4). Formation of complex SN5 and, to a lesser extent, SN3, was nonspecifically inhibited by immune (Figure 11 lanes 3–5) as well as non-immune (Figure 11 lane 6) serum. Thus the data from competitive binding and binding inhibition assays indicated that, in non-immortalized human astrocytes, AP-1-like proteins can bind to one or both of the upstream AP-1 binding motifs present in LTR region nt −354 to −316.

Discussion

Our studies demonstrate that CMV and HHV-6 infection similarly enhance the transient replication of HIV-1 in nonimmortalized, low passage astrocytes cultured from human fetal brain and rigorously monitored during serial passage to exclude fibroblast contamination (McCarthy *et al*, 1995). However, HHV-6 or CMV infection did not convert this transient HIV-1 gene expression into a productive infection in astrocytes. The enhancement of HIV-1 replication may be potentiated by *trans*-activation of the HIV-1 promoter, the LTR. On this point, our studies confirm and extend previous observations of CMV-mediated *trans*-activation of the HIV-1 major promoter in astroglial cells, including tumor-derived (Koval *et al*, 1995; Duclos *et al*, 1989) and nonimmortalized (Ho *et al*, 1991) cultures. However, this is the first reported demonstration of HHV-6 infection mediating *trans*-activation of HIV LTR in nonimmortalized, low passage human fetal astrocytes. The findings in this study indicate that, during CMV or HHV-6 infection in human fetal astrocytes, *trans*-activation of the LTR is mediated by diverse molecular interactions

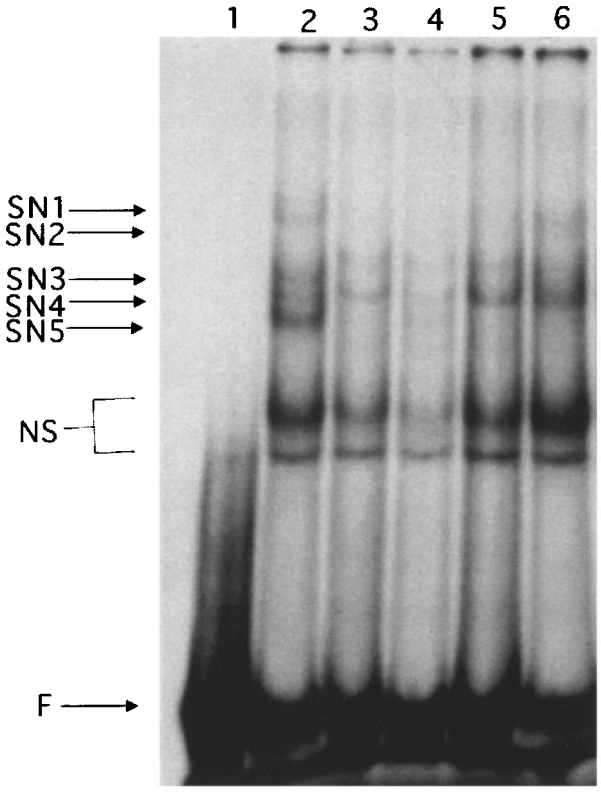


Figure 11 EMSA using probe SN in the presence of antibodies specific for the Jun or Fos family of transcription factors. Probe SN was reacted with nuclear protein extracts from HHV-6-infected astrocytes in the absence (lane 2) or the presence of specific antibodies (lanes 3–5) or non-immune serum (lane 6). Lane 3 contains antibody reactive with c-Fos p62; lanes 4 and 5 contain broadly reactive antibodies against Fos and Jun proteins, respectively.

involving cellular factors such as NFκB, Sp-1, and AP-1 plus multiple potential response sites on the LTR upstream of a functional TATA box.

Several functionally deficient LTR mutant constructs were *trans*-activated to varying degrees during CMV or HHV-6 infection in astrocytes. Functional deletion of any one of the core, enhancer, or upstream (nt −360 to −147) modulatory elements in the LTR was partially compensated by the presence of the others during *trans*-activation by HHV-6 or CMV infection, even though mutation of any one of these sites substantially weakened the basal expression level of the LTR in astrocytes. In particular, the modulatory sequences upstream of the *Ava*I site at nt −147 were important to positive regulation of both basal and HHV-6- or CMV-mediated *trans*-activation of LTR expression in human fetal astrocytes. This contrasted with the reported negative regulation by this region of LTR expression in T-lymphocytes (Horvat *et al*, 1991). A minimal promoter consisting of only the TATA box and TAR regions was not effectively *trans*-activated by either CMV or HHV-6. Additional regulatory sequences between nt −57 to −30 were required for any significant *trans*-activation of the LTR. Interestingly, this region contains the most proximal Sp1-binding site, which has been implicated in synergistic activation of the LTR with COUP-TF in microglial cells (Rohr *et al*, 1997). Our studies with both CMV and HHV-6 infection are consistent with several studies of CMV-LTR interactions in human fibroblasts that implicate multiple regions of the LTR in CMV-*trans*-activated LTR expression, including TAR (Barry *et al*, 1990) and upstream elements (Ghazal *et al*, 1991; reviewed in Ghazal and Nelson, 1993). However, HHV-6-*trans*-activated LTR expression in T-lymphocytes (Horvat *et al*, 1991; Geng *et al*, 1992) or CV-1 monkey kidney cells (Zhou *et al*, 1994) has been reported to be critically dependent on a functional NFκB enhancer element. Thus, when compared to other cell types, the pattern of *trans*-activated LTR activity in human fetal astrocytes reflects the influence of the host cell on molecular interactions between activating factors and the LTR.

Recent studies of transcription factor binding in both the enhancer (Krebs *et al*, 1997) and the upstream (−480 to −160) modulatory region (Canonne-Hergaux *et al*, 1995) of the HIV LTR indicate that there are cell-type specific and HIV-1 strain-specific differences in nuclear protein binding to sequence elements in these regions. Our electrophoretic mobility shift studies confirmed that core, enhancer, and upstream modulatory regions of the HIV-1 strain NL4-3 LTR can interact specifically with nuclear proteins from both uninfected and HHV-6- or CMV-infected human fetal astrocytes. Competition studies identified DNA-nuclear protein complexes with NFκB-, Sp1-, or AP-1-type binding motifs. The mobility shift studies did not detect DNA-protein complexes that were unique to HHV-6 or CMV infection. But the relative proportion of certain DNA-protein complexes appeared to increase in nuclear protein extracts from infected cells compared to those from their respective mock-infected controls. These relatively more abundant complexes included those with Sp1- or AP-1-type binding. This relative enhancement of nuclear protein binding is a plausible mechanism to explain the functional (CAT expression) data suggesting multiple response regions mediate CMV-LTR or HHV-6-LTR interactions in human fetal astrocytes. The pattern of DNA-protein complexes observed with our probe SN and the various nuclear extract preparations from human fetal astrocytes is very similar to the pattern observed by Canonne-Hergaux *et al* (1995) with nuclear proteins from astrocytoma cells and the LTR sequence from HIV-1 strain LAI, the prototype lymphotropic strain. Canonne-Hergaux *et al* determined that most of the astrocytoma nuclear proteins forming complexes with the LTR sequence within

this region (nt −354 to −316) belong to the nuclear receptor family and the COUP-TF sub-family of factors. They were unable to demonstrate competitive inhibition of these DNA-protein complexes by the consensus AP-1 binding motif TGACTCA. We did detect AP-1 like protein binding to the putative nuclear receptor-responsive region (nt −356 to −316) within the modulatory region of the LTR using nuclear extracts from mock-infected, HHV-6-infected, or CMV-infected astrocytes. The differences in nuclear protein binding may reflect cell type-specific differences in LTR-driven transcription (Canonne-Hergaux *et al*, 1995). The nuclear receptor-responsive element of the LTR, spanning approximately the −356 to −320 sequence, may have positive or negative effects on LTR activity, depending on cell type (Ladias, 1994). Moreover, there may be cell-type specific forms of transcription factors such as AP-1 (Canonne-Hergaux *et al*, 1995) or NFκB (Taylor *et al*, 1994) that influence LTR expression in astroglial cells. Most studies of HIV LTR activity in 'astroglial' cells have used growth transformed astroglial tumor cell lines such as U373MG (Duclos *et al*, 1989; Koval *et al*, 1995) or TH4-7-5 (Brack-Werner *et al*, 1992). Arguably, these immortalized astrocytes are more likely to have different endogenous levels of cellular transcription factors or regulatory gene expression associated with immortalized growth, which are different from non-immortalized astrocytes. Thus non-immortalized astrocyte cultures may exhibit fine differences from immortalized astrocytoma cells in the molecular events and factors that regulate HIV expression and Herpesvirus-HIV interactions.

Herpesvirus-mediated activation of the HIV LTR in dually infected (HIV plus HHV-6 or HIV plus CMV) astrocytes is a plausible mechanism whereby latent or 'restricted' HIV infection in these cells could be enhanced or activated. Then astrocytes would function as a reservoir of HIV in the brain which, when activated, would contribute to increased viral load in the CNS. There is some controversy over whether transient expression assays of CMV-mediated *trans*-activation of the HIV LTR are predictive of CMV-mediated effects on HIV infection (Jault *et al*, 1994; Spector *et al*, 1994). After evaluating the interactions between CMV and HIV-1 in several human brain-derived neoplastic cell lines, Jault *et al* (1994) proposed that, in cells wherein both CMV and HIV established productive infection, CMV inhibited HIV replication (Spector *et al*, 1994). However, CMV-mediated activation of HIV-1 could occur in neural cells wherein HIV-1 gene expression is limited; this is precisely the expected scenario in HIV-infected astrocytes. In agreement with this, we observed that both CMV and HHV-6 transiently enhanced HIV replication in our human fetal astrocyte cultures. In the context of a neuropathogenetic cascade mediated by glial and immune cells in the CNS, even a modest increase in HIV gene products resulting from direct HHV-6-HIV LTR or CMV-HIV LTR interactions could accelerate the progression of HIV-related CNS disease. Thus HHV-6 or CMV can act through the astrocyte 'reservoir' to increase AIDS neurological morbidity.

Materials and methods

Preparation of human fetal astrocyte cultures

All cultures were prepared from first trimester human fetal specimens of 46–83 days gestation. Fetal CNS tissue was obtained from the Human Embryology Laboratory, University of Washington (Seattle, WA). Procedures for procurement and use of this human fetal CNS tissue were approved and monitored by the University of Miami School of Medicine's Medical Sciences Subcommittee for the Protection of Human Subjects. The CNS tissue from each fetal specimen was processed separately and independently, as were subsequent cell cultures; there was no pooling of CNS tissues from distinct fetal specimens. Highly enriched human fetal astrocyte cultures were prepared as previously reported (McCarthy *et al*, 1995). Briefly, CNS tissue consisting primarily of rhombencephalon and mesencephalon was transferred to culture medium, approximately 2 ml per 500 mg tissue. Culture medium consisted of Dulbecco's Modified Eagle's Medium (DMEM) plus Ham's F12 medium (F12) in equal proportions supplemented with 10% (v/v) fetal bovine serum (DF-FBS). Tissue was mechanically dissociated by repeated trituration (up to six passages) with a 1 ml serological pipette followed by a glass Pasteur pipette. The cell suspension was further diluted in DF-FBS and plated onto poly-L-lysine (Sigma Chemical, St. Louis, MO)-coated tissue culture dishes. To avoid fibroblast overgrowth, cultures were adapted to serum-free B16 medium (Brewer and Cotman, 1989) supplemented with 5 ng/ml basic fibroblast growth factor (FGF-2). For serial passage, cells were subcultured when confluent, approximately once per week. Infection or transfection studies were performed with cultures serially passaged between 3 and 6 times. Cultures were monitored by immunofluorescence assay (IFA) for expression of glial fibrillary acidic protein (GFAP), a marker for astrocytes, and fibronectin or prolyl-4-hydroxylase for fibroblast-type cells (McCarthy *et al*, 1995).

Viral infection of astrocytes

Human fetal astrocyte cultures were infected with cell-free preparations of human CMV strain AD169 as previously described (McCarthy *et al*, 1995). CMV stocks were grown in human lung fibroblasts (MRC-5) in DMEM supplemented with 2% (v/v) FBS. Astrocytes (10^6 cells) were infected at a multiplicity of infection (moi) of 0.1 plaque forming units (p.f.u.) per cell approximately 14–16 h prior

to transfection with plasmids or at an moi of 1 p.f.u./cell 8 h post transfection. Using either of these infection conditions, CMV antigen was present in greater than 80% of astrocytes in the culture by 48 h post transfection as confirmed by IFA for viral structural antigens (McCarthy *et al*, 1995). In parallel, replicate astrocytes were 'mock-infected' with DMEM-2% FBS. Viral-infected or mock-infected cultures were maintained in DMEM supplemented with FBS as previously described (McCarthy *et al*, 1995). CMV-infected monolayers remained intact and viable for 8–10 days.

HHV-6 type A strain GS was grown in the HSB2 human T-lymphoblastoid cell line (He *et al*, 1996). HHV-6(GS)-infected HSB2 cells were cultured to a titer of approximately 10 TCID50/cell by feeding GS-infected cells with varying proportions of uninfected HSB2 cells. To infect astrocytes, approximately 2×10^5 GS-infected HSB2 cells were co-cultivated with 2×10^6 astrocytes for 12 h starting 8 h post transfection, for an moi of approximately 1 $TCID_{50}$/astrocyte. In parallel, replicate astrocytes were 'mock-infected' by co-cultivation with an equal number of uninfected HSB2 cells. After the 12 h co-cultivation interval, all astrocyte monolayers were washed multiple times with phosphate-buffered saline (PBS) to remove all HSB2 cells, then further incubated in DMEM-2% FBS. HHV-6 antigen was present in 50–70% of cells in the culture by 48 h post transfection as confirmed by IFA for viral antigens (He *et al*, 1996). HHV-6-infected monolayers remained intact and viable for 7–9 days.

Recombinant plasmids and transfections

Recombinant plasmids used to assay promoter function included the HIV-1 LTR, the Rous Sarcoma Virus (RSV) LTR, or the CMV immediate early (IE) promoter linked to the CAT or β-galactosidase reporter genes. These plasmids are designated HIV LTR-CAT, RSV-CAT, CMV-CAT, LTR-β-gal and CMV-β-gal respectively. Plasmid LTR-CAT was the HIV LTR-CAT 7 construct (a gift of Dr. Lung-Ji Chang, Univ. of Alberta, Canada), which contained both the 3′ untranslated regions (U3) and the repeat sequences (R) of the LTR. It was derived by ligation of the 3′ LTR sequence from lymphotropic HIV-1 strain NL4-3 with the CAT gene of the pSV2CAT construct into the pT7T318U vector (Pharmacia Biotech, Piscataway, NJ). Plasmids RSV-CAT (Yamamoto *et al*, 1980) and CMV-CAT (McCarthy *et al*, 1995) have been previously described. Construction of the mutant LTR-CAT plasmids (Figure 4A) has been described in detail (Geng *et al*, 1992; Zhou *et al*, 1994). Briefly, the −29LTR has a deletion of all regulatory elements on the LTR excepting the TATA box and TAR regions. The −57LTR contains one Sp1 site and the TATA box. The −147LTR contains the NFκB sites, the Sp1 sites, the TATA box, and the TAR region, but the negative regulatory element (NEG) is deleted. Additional mutant plasmids include one deleted of all Sp1 sites (dlSp1 LTR) and one with mutations at both NFκB sites (mutant NFκB LTR) that eliminate *trans*-activation of the HIV LTR by NFκB protein (Mosca *et al*, 1987). Mutant LTR-CAT constructs were always assayed in parallel with the intact LTR-CAT construct. A recombinant plasmid containing the HIV-1 *tat* gene under the control of the Rous Sarcoma Virus promoter (RSV-tat) was constructed by replacing the CAT gene in the RSV-CAT construct (Yamamoto *et al*, 1980) with the *Sal*I–*Kpn*I fragment of HIV-1 (Jung and Wood, 1991). This plasmid was co-transfected into astrocytes with LTR-CAT to determine the effect of tat on LTR activity in the astrocytes. To verify the function of the RSV promoter itself in human fetal astrocytes, the activity of the RSV promoter in transfected astrocytes was compared to that of the HIV LTR and the CMV IE promoters as determined by CAT expression directed by these promoters. Basal CAT expression driven by HIV LTR and RSV promoters (HIV LTR-CAT and RSV-CAT constructs respectively) was equivalent, and approximately fivefold less than CAT expression driven by the CMV IE promoter (CMV-CAT construct) in parallel cultures of transfected human fetal astrocytes (data not shown). The CAT gene in LTR-CAT was replaced by the β-galactosidase gene to generate the plasmid LTR-βgal. β-galactosidase expression in transfected cells was determined by IFA or enzyme assay. Approximately 2–3% of astrocytes expressed detectable antigen 72 h after transfection with the LTR-β-gal plasmid under transient gene expression conditions described below (data not shown). Plasmid CMV-CAT was constructed by replacing the LTR of the LTR-CAT construct with the CMV IE promoter (kindly provided by Dr Lung-Ji Chang, University of Alberta, Alberta, Canada). The CAT gene in CMV-CAT was replaced by the β-galactosidase gene to generate the plasmid CMV-βgal. CMV-CAT expression was assayed as an external positive control for variability among astrocyte cultures in transient gene expression assay efficiency. CMV-β-gal expression was assayed as an internal control for transfection efficiency.

Plasmid DNA was transfected into cells using cationic liposomes (Lipofectamine, GIBCO BRL, Gaithersburg, MD) mixed with 6 μg total plasmid DNA per 10^6 cells in multi-well plates. In co-transfections, 2 μg LTR-CAT plus 4 μg RSV-tat plasmids were mixed together with cationic liposomes. This combination gave maximal tat-activated LTR-CAT expression in all astrocyte cultures. Comparison and control cultures were transfected with 4 μg carrier plasmid (pBR322) DNA plus 2 μg LTR-CAT DNA per 10^6 cells to maintain a constant total amount (6 μg) of transfected DNA in all LTR-transfected cultures. DNA-liposome mixtures were incubated with cultures for 8 h in serum-free

DMEM, then cultures were washed and maintained in DMEM-FBS. Transient LTR activity in transfected human fetal astrocytes was detectable as early as 24 h post transfection, with maximal activity measured 48–72 h post transfection, followed by a decline in activity by 96 h post transfection (data not shown). Thus, transfected cultures were harvested 72 h after transfection for reporter gene assays as previously described (McCarthy *et al*, 1995). The time course of transient LTR expression was not changed by concurrent CMV or HHV-6 infection or by co-transfection of the RSV-tat plasmid (data not shown). Lysate protein prepared from harvested cultures was quantitated by the BCA protein assay (Pierce, Rockford, IL) using bovine serum albumin as a standard. LTR-CAT expression was quantitated as pg CAT protein per 5 μg lysate protein determined with a commercial ELISA assay (Boehringer Mannheim, Indianapolis, IN) which measures CAT protein rather than CAT enzyme activity. This method had been found comparable in efficiency and sensitivity to the CAT enzyme assay (McCarthy *et al*, 1995). Basal LTR-CAT expression is defined as LTR-CAT expression in untreated astrocytes (Figure 2B) or in mock-infected astrocytes in the absence of HHV-6 or CMV co-infection or RSV-tat plasmid co-transfection.

To study the effect of HHV-6 or CMV on replication of HIV-1 in astrocytes, the proviral DNA clone of HIV-1 lymphotropic strain NL4-3 (Adachi *et al*, 1986) was transfected into astrocytes either before or after CMV(AD169) or HHV-6(GS) infection of astrocytes as described above for the LTR constructs. Astrocytes were transfected with cationic liposomes and 1 μg proviral DNA per 3–4×10^5 cells. Transfected or *mock-transfected* culture supernatants were collected every 48 h with change of culture medium for measurement of soluble p24 production by ELISA (Immunotech Inc, Westbrook, ME, USA). The pNL4-3 clone was obtained from the NIH AIDS Reference and Reagent Program (Rockville, MD, USA).

Preparation of nuclear extracts and electrophoretic mobility shift assays (EMSA)

Astrocytes were cultured in 225 cm^2 flasks to confluence. A total of 2×10^8 cells were harvested for each nuclear extract preparation. Astrocytes were mock-infected, HHV-6-infected, or CMV-infected in parallel as described above and then harvested at 72 h post infection. Nuclear extracts were prepared by a modification of the method of Dignam *et al* (1983). Monolayers were harvested by scraping cells into ice cold PBS and parallel cultures were normalized to obtain equal numbers of cells for nuclear extract preparations. All subsequent steps were carried out at 4°C. After initial centrifugation, cells were resuspended in 3 ml low salt buffer (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 0.5 mM DTT, 10 mM KCl) per 2×10^8 cells and allowed to stand for 20 min at 4°C; then NP-40 was added to a final concentration of 0.8% (v/v). The homogenate was monitored microscopically to confirm cell lysis and release of intact nuclei. Cell debris was then removed from the lysate by low speed centrifugation. Nuclei were then pelleted from the lysate by high speed centrifugation, and the nuclear pellet was suspended into 1 ml of high salt buffer (20 mM HEPES pH 7.9, 25% glycerol (v/v), 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF) per 2×10^8 cells. After 30 min incubation at 0°C, the nuclear lysate was centrifuged for 30 min at high speed. The supernatant was collected and designated as nuclear extract, dialyzed overnight at 4°C against 300 ml of dialysate buffer (20 mM HEPES pH 7.9, 20 mM KCl, 1 mM $MgCl_2$, 2 mM DTT, 17% glycerol). The nuclear extract was recovered from dialysis tubing and NP40 was added to a final concentration of 1% (v/v). Nuclear extract protein concentration was quantitated by the BCA protein assay (Pierce, Rockford, IL).

Three DNA oligonucleotide probes were constructed for EMSA of astrocyte nuclear protein binding to the LTR (Figure 7). Nucleotide sequences used were those reported for the complete genome of HIV-1 strain NL4-3 (GenBank Accession M19921). Probes I and II were purified from restriction enzyme digests of the U3 region of the LTR-CAT 7 plasmid. Probe I (nt −158 to +5) is the *Ava*I-*Bgl*II LTR fragment; it contains both core and enhancer regions of the LTR. Probe II (nt −340 to −159) is the *Eco*RV–*Ava*I LTR fragment; it contains the upstream modulatory regions of the LTR. Probes I and II were labeled with [α-^{32}P]ATP and Klenow enzyme. Probe SN (nt −354 to −316) was synthesized as two complementary DNA single strands on an automated nucleotide synthesizer (Model 394, Applied Biosystems, Perkin Elmer, Foster City, CA). The single strands were mixed, heated to 95°C, and re-annealed overnight prior to radiolabeling with α-^{32}P-CTP and Klenow enzyme.

Protein-DNA binding reactions were performed with 5–10 μg nuclear protein extracts and 30 000–50 000 c.p.m. ^{32}P end-labeled probe in a binding buffer containing 10 mM Tris pH 7.5, 50 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, 5% (v/v) glycerol (all from Sigma Chemicals, St. Louis, MO), and 0.1–0.5 μg poly(dI-dC) (Pharmacia Biotech, Piscataway, NJ). Mixtures were incubated for 30 min at room temperature, then sucrose was added to a final concentration of 4% (v/v). The reaction mixtures were analyzed by non-denaturing gel electrophoresis on 5% acrylamide gels in 0.25 × Tris-borate-EDTA buffer, pH 8.0. Following electrophoresis the gels were dried and the protein-DNA complexes visualized by autoradiography. For competition binding assays, double-stranded oligonucleotides containing consensus NFκB-, Sp1, or AP-1 binding motifs were obtained from Promega (Madison, WI). For binding inhibition assays, 3 μg antibodies directed against c-Jun p39 proteins or

against c-Fos p62 proteins (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were mixed with nuclear proteins for 30 min at 4°C prior to addition of radiolabeled probes. These antibodies included a polyclonal rabbit antibody broadly reactive with c-Jun, Jun B and Jun D p39 proteins, a mouse monoclonal antibody broadly reactive with c-Fos, Fos B, Fra-1, and Fra-2 proteins, and a polyclonal rabbit antibody reactive only with c-Fos p62 protein.

Acknowledgements

This study was supported in part by the Office of Research and Development (R&D), Medical Research Service, Department of Veterans Affairs; by Pediatric AIDS Foundation Pediatric Scholar Awards 770222-14-PF and 77272-18-PFR to M.Mc., and by Public Health Service grants AI30356 and CA62810 to C.W. M.M. was a Pediatric AIDS Foundation Scholar. Larisa Fedoseyeva provided technical assistance with transient gene expression assays. Allan Prejusa provided technical assistance with gel shift assays. The proviral clone pNL4-3 was obtained from Dr Malcom Martin through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. We thank Dr Scott Whittemore for valuable advice and suggestions throughout this study.

References

Achim CL, Wang R, Miners DK, Wiley CA (1994). Brain viral burden in HIV infection. *J Neuropathol Exp Neurol* **53:** 284–294.

Adachi A, Gendelman HE, Koenig S, Folks T, Willey R, Rabson A, Martin MA (1986). Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. *J Virol* **59:** 284–291.

Barry PA, Pratt-Lowe E, Peterlin BM, Luciw PA (1990). Cytomegalovirus activates transcription directed by the long terminal repeat of human immunodeficiency virus type 1. *J Virol* **64:** 2932–2940.

Barry PA, Pratt-Lowe E, Unger RE, Luciw PA (1991). Cellular factors regulate transactivation of human immunodeficiency virus type 1. *J Virol* **65:** 1391–1399.

Biegalke BJ, Geballe AP (1991). Sequence requirements for activation of the HIV-1 LTR by human cytomegalovirus. *Virology* **183:** 381–385.

Blumberg BM, Gelbard HA, Epstein LG (1994). HIV-1 infection of the developing nervous system: central role of astrocytes in pathogenesis. *Virus Res* **32:** 253–267.

Brack-Werner R, Kleinschmidt A, Ludvigsen A, Mellert W, Neumann M, Herrmann R, Khim MC, Burny A, Muller-Lantzsch N, Stavrou D (1992). Infection of human brain cells by HIV-1: restricted production in chronically infected human glial cell lines. *AIDS 6:* 273–285.

Brewer GJ, Cotman CW (1989). Survival and growth of hippocampal neurons in defined medium at low density: advantages of a sandwich culture technique or low oxygen. *Brain Res* **494:** 65–74.

Canonne-Hergaux F, Aunis D, Schaeffer E (1995). Interactions of the transcription factor AP-1 with the long terminal repeat of different human immunodeficiency virus type 1 strains in Jurkat, glial, and neuronal cells. *J Virol* **69:** 6634–6642.

Dignam JD, Lebovitz RM, Roeder RG (1983). Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids* **11:** 1475–1489.

Duclos H, Elfassi E, Michelson S, Arenzana-Seisdedos F, Munier A, Virelizier J-L (1989). Cytomegalovirus infection and trans-activation of HIV-1 and HIV-2 LTRs in human astrocytoma cells. *AIDS Res Hum Retro* **5:** 217–224.

Fiala M, Mosca JD, Barry P, Luciw PA, Vinters HV (1991). Multi-step pathogenesis of AIDS - role of cytomegalovirus. *Res Immunol* **142:** 87–95.

Gendelman HE, Phelps W, Feigenbaum L, Ostrove JM, Adachi A, Howley PM, Khoury G, Ginsberg HS, Martin MA (1986). Trans-activation of the human immunodeficiency virus long terminal repeat sequence by DNA viruses. *Proc Natl Acad Sci USA* **83:** 9759–9763.

Geng Y, Chandran B, Josephs SF, Wood C (1992). Identification and characterization of a HHV-6 gene segment that transactivates the HIV-1 promoter. *J Virol* **66:** 1564–1570.

Ghazal P, Nelson JA (1993). Interactions between cytomegalovirus immediate-early proteins and the long terminal repeat of human immunodeficiency virus. *Rev Med Virol* **3:** 47–55.

Ghazal P, Young J, Guilietti E, DeMattei C, Garcia J, Gaynor R, Stenberg RM, Nelson JA (1991). A discrete *cis* element in the human immunodeficiency virus long terminal repeat mediates synergistic *trans* Activation by cytomegalovirus immediate-early proteins. *J Virol* **65:** 6735–6742.

Harrison MJG, McArthur JC (1995). In: *AIDS and Neurology*. London: Churchill Livingstone; pp 136–141.

He J, McCarthy M, Zhou Y, Chandran B, Wood C (1996). Infection of primary human fetal astrocytes by human herpesvirus 6. *J Virol* **70:** 1296–1300.

Ho W-Z, Long L, Douglas SD (1991). Human cytomegalovirus infection and trans-activation of HIV-1 LTR in human brain-derived cells. *J Acq Immune Defic Synd* **4:** 1098–1106.

Horvat RT, Josephs SF, Wood C, Balachandran N (1991). Transactivation of HIV promoter by human herpesvirus-6 strains GS and Z-29 in primary human T lymphocytes and identification of transactivating HHV-6(GS) gene fragments. *J Virol* **65:** 2895–2902.

Jault FM, Spector SA, Spector DH (1994). The effects of cytomegalovirus on human immunodeficiency virus replication in brain-derived cells correlate with permissiveness of the cells for each virus. *J Virol* **68:** 959–973.

Johnson RT, Glass JD, McArthur JC, Chesebro BW (1996). Quantitation of human immunodeficiency virus in brains of demented and nondemented patients with acquired immunodeficiency syndrome. *Ann Neurol* **39:** 392–395.

Jung M, Wood C (1991). Activation of retroviral promoter by trans-acting factors. *Virol (Life Sci Adv)* **10:** 77–88.

Koval V, Jault FM, Pal PG, Moreno TN, Aiken C, Trono D, Spector SA, Spector DH (1995). Differential effects of human cytomegalovirus on integrated and unintegrated human immunodeficiency virus sequences. *J Virol* **69:** 1645–1651.

Krebs FC, Goodenow MM, Wigdahl B (1997). Neuroglial ATF/CREB factors interact with the human immunodeficiency virus type 1 long terminal repeat. *J NeuroVirol* **3(S1):** S28–S32.

Ladias JAA (1994). Convergence of multiple nuclear receptor signaling pathways onto the long terminal repeat of human immunodeficiency virus-1. *J Biol Chem* **26:** 5944–5951.

Laurence J (1990). Molecular interactions among Herpesviruses and human immunodeficiency viruses. *J Inf Dis* **62:** 338–346.

Lawrence GL, Chee M, Craxton MA, Gompels UA, Honess RW, Barrell BG (1990). Human herpesvirus-6 is closely related to cytomegalovirus. *J Virol* **64:** 287–299.

Lipton SA, Gendelman HE (1995). Dementia associated with the acquired immunodeficiency syndrome. *New Engl J Med* **332:** 934–940.

Lusso P, Ensoli B, Markham PD, Ablashi DV, Salahuddin SZ, Tschachler E, Wong-Staal F, Gallo RC (1989). Productive dual infection of human CD4+ T lymphocytes by HIV-1 and HHV-6. *Nature (London)* **337:** 370–373.

Lusso P, Gallo RC (1995). Human herpesvirus-6 in AIDS. *Immunol Today* **16:** 67–71.

Martin MED, Nicholas J, Thomson BJ, Newman C, Honess RW (1991). Identification of a transactivating function mapping to the putative immediate-early locus of human herpesvirus-6. *J Virol* **65:** 5381–5390.

McCarthy M, Wood C, Fedoseyeva L, Whittemore SR (1995). Media components influence viral gene expression assays in human fetal astrocyte cultures. *J NeuroVirol* **1:** 275–285.

McCarthy M, He J, Wood C (1998). HIV-1 strain-associated variability in infection of primary neuroglia. *J NeuroVirol* **4:** 80–89.

Mosca JD, Bednarik DP, Raj NBK, Rosen CA, Sodroski JG, Haseltine WA, Hayward GS, Pitha PM (1987). Activation of human immunodeficiency virus by herpesvirus infection: Identification of a region within the long terminal repeat that responds to a trans-acting factor encoded by herpes simplex virus 1. *Proc Natl Acad Sci USA* **84:** 7408–7412.

Nabel GJ, Baltimore D (1987). An inducible transcription factor activates expression of human immunodeficiency virus in T cells. *Nature (London)* **326:** 711–713.

Nelson JA, Reynolds-Kohler C, Oldstone MBA, Wiley CA (1988). HIV and HCMV coinfect brain cells in patients with AIDS. *Virology* **165:** 286–290.

Rohr O, Sawaya BE, Aunis D, Schaeffer E (1997). Regulation of HIV-1 gene transcription by the nuclear receptor COUP-TF in human brain cells. *J NeuroVirol* **3(S1):** S96.

Saito Y, Sharer LR, Dewhurst S, Blumberg BM, Hall CB, Epstein LG (1995). Cellular localization of human herpesvirus-6 in the brains of children with AIDS encephalopathy. *J NeuroVirol* **1:** 30–39.

Saito Y, Sharer LR, Epstein LG, Michaels J, Mintz M, Louder M, Golding K, Cvetkovich TA, Blumberg BM (1994). Overexpression of nef as a marker for restricted HIV-1 infection of astrocytes in postmortem pediatric central nervous tissues. *Neurology* **44:** 474–480.

Sawaya BE, Rohr O, Filliol D, Aunis D, Schaeffer E (1996). Regulation of HIV-1 gene expression by COUP-TF and retinoic acid receptors in human brain cells. *J NeuroVirol* **2:** 19.

Sissons JGP, Borysiewicz LK, Rodgers B, Scott D (1986). Cytomegalovirus - its cellular immunology and biology. *Immunol Today* **7:** 57–61.

Spector DH, Koval V, Jault FM, Lathey J, Spector SA (1994). Positive and negative effects of human cytomegalovirus on HIV replication. In: *Interactions between retroviruses and herpesviruses*. Kung HJ, Wood C. (eds.) Singapore: World Scientific Publishing Co. pp 65–89.

Sodroski J, Rosen C, Wong-Staal F, Salahuddin SZ, Popovic M, Arya S, Gallo RC, Haseltine WA (1985). Trans-acting transcriptional regulation of human T-cell leukemia virus type III long terminal repeat. *Science* **227:** 171–173.

Spencer DC, Price RW (1992). Human immunodeficiency virus and the central nervous system. *Annual Rev Microbiol* **46:** 655–693.

Takahashi K, Sonoda S, Higashi K, Kondo T, Takahashi H, Takahashi M, Yamanishi Y (1989). Predominant CD4+ lymphocyte tropism of human herpesvirus-6-related virus. *J Virol* **63:** 3161–3163.

Taylor JP, Pomerantz RJ, Raj GV, Kashanchi F, Brady JN, Amini S, Khalili K (1994). Central nervous system-derived cells express a κB-binding activity that enhances human immunodeficiency virus type 1 transcription in vitro and facilitates TAR-independent transactivation by tat. *J Virol* **68:** 3971–3981.

Tornatore C, Chandra R, Berger JR, Major EO (1994a). HIV-1 infection of subcortical astrocytes in the pediatric central nervous system. *Neurology* **44:** 481–487.

Tornatore C, Meyers K, Atwood W, Conant K, Major EO (1994b). Temporal patterns of human immunodeficiency virus type 1 transcripts in human fetal astrocytes. *J Virol* **68:** 93–102.

Tornatore C, Nath A, Amemiya K, Major EO (1991). Persistent human immunodeficiency virus type 1 infection in human fetal glial cells reactivated by T-cell factor(s) or by the cytokines tumor necrosis factor alpha and interleukin-1 beta. *J Virol* **65:** 6094–6100.

Walker S, Hagemeier C, Sissons JGP, Sinclair JH (1992). A 10-base-pair element of the human immunodeficiency virus type 1 long terminal repeat (LTR) is an absolute requirement for transactivation by the human cytomegalovirus 72-kilodalton IE1 protein but can be compensated for by other LTR regions in transactivation by the 80-kilodalton IE2 protein. *J Virol* **66:** 1543–1550.

Yamamoto T, Jay G, Pastan I (1980). Unusual features in the nucleotide sequence of a cDNA clone derived from the common region of avian sarcoma virus messenger RNA. *Proc Natl Acad Sci USA* **77:** 176–180.

Zhou Y, Chang CK, Qian G, Chandran B, Wood C (1994). *trans*-Activation of the HIV promoter by a cDNA and its genomic clones of human herpesvirus-6. *Virology* **199:** 311–322.