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Short Communication

Transcriptional activity of blood- and cerebrospinal fluid–derived *nef*/long-terminal repeat sequences isolated from a slow progressor infected with *nef*-deleted human immunodeficiency virus type 1 (HIV-1) who developed HIV-associated dementia

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The authors studied the transcriptional activity of blood- and cerebrospinal fluid (CSF)-derived *nef*/long-terminal repeat (LTR) sequences isolated from a slow progressor infected with *nef*-deleted human immunodeficiency virus type 1 (HIV-1) who developed HIV-associated dementia (HIVD). The transcriptional activity of CSF-derived *nef*/LTR clones isolated during HIVD was up to 4.5-fold higher than blood-derived clones isolated before and during HIVD when tested under basal, phorbol 12-myristate 13-acetate- (PMA-), and Tat-activated conditions, and was associated with the presence of duplicated nuclear factor (NF)-κB and specificity factor-1 (Sp-1) binding sites coupled with a truncated *nef* sequence, increased replication capacity, and high CSF viral load. Thus, *nef* and LTR mutations that augment transcription may contribute to neuropathogenesis of *nef*-deleted HIV-1. *Journal of NeuroVirology* (2006) **12,** 219–228.

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The *nef* gene is a major determinant of virulence in primate lentiviruses. Strains of simian immunodeficiency virus (SIV) with mutations in *nef* have attenuated replication capacity and pathogenicity, compared with wild type SIV strains (Chakrabarti *et al*, 1995; Desrosiers *et al*, 1998; Hofmann-Lehmann *et al*, 2003; Iafrate *et al*, 2000; Kestler *et al*, 1991; Messmer *et al*, 2000). Similarly, mutations in the *nef* gene of human immunodeficiency virus type 1 (HIV-1) may promote long-term nonprogression or slow progression of HIV-1 infection in humans (Deacon *et al*, 1995; Kirchhoff *et al*, 1995; Kondo *et al*, 2005; Mariani *et al*, 1996; Rhodes *et al*, 2000). The largest described cohort of long-term survivors infected with *nef*-deleted HIV-1 is the Sydney blood bank cohort (SBBC), which consists of multiple individuals who became infected with an attenuated strain of HIV-1 via contaminated blood products from a common blood donor between 1981 and 1984 (Deacon *et al*, 1995; Learmont *et al*, 1992, 1999). Viral attenuation has been attributed to

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gross deletions in the HIV-1 *nef*/long-terminal repeat (LTR) region of the HIV-1 genome (Deacon *et al*, 1995). The presence of duplicated and/or rearranged nuclear factor (NF)-κB and Sp-1 binding sites in the LTR are also unique properties of viral strains harbored by SBBC members (Churchill *et al*, 2004, 2006; Deacon *et al*, 1995). Despite being infected from a single source, SBBC members comprise slow progressors and long-term nonprogressors (Churchill *et al*, 2006). The SBBC therefore provides a unique opportunity to study the pathogenesis of *nef*-deleted HIV-1 in a naturally occurring human setting.

HIV-1 invades the central nervous system (CNS) early in the course of infection and causes HIV-associated dementia (HIVD) or a milder form of neurocognitive impairment termed minor cognitive motor disorder in 10% to 20% of patients with acquired immunodeficiency syndrome (AIDS) (reviewed in Dunfee *et al*, 2006; Gabuzda *et al*, 2002; Gonzalez-Scarano and Martin-Garcia, 2005). Features of HIVD include cognitive slowing and memory impairment typically progressing to frank dementia and death within a year of onset. CNS infection can cause subacute encephalitis, characterized by reactive astrocytes, white matter abnormalities, microglial nodules, perivascular inflammation, multinucleated giant cells, and neuronal cell loss (Dunfee *et al*, 2006; Gabuzda and Wang, 2000). Highly active antiretroviral therapy (HAART) reduces cerebrospinal fluid (CSF) HIV-1 RNA levels and improves neurocognitive function and CNS abnormalities detected by neuroimaging studies (Sacktor *et al*, 2002). However, relatively poor CNS penetration by most drugs used in HAART contributes to the development of resistance mutations and compartmentalized evolution of HIV-1 in the CNS (Smit *et al*, 2004; Strain *et al*, 2005).

Although the CNS is a well-characterized anatomical reservoir of HIV-1, the ability of *nef*-deleted HIV-1 strains to persist in the CNS and cause neurologic disease is unclear. Previous studies of SIV or chimeric simian-human immunodeficiency virus (SHIV) with mutations in *nef* showed diminished neurotropism in macaque models (Singh *et al*, 2002; Thompson *et al*, 2003). By analogy, *nef* is considered an important factor in the neuropathogenesis of HIV-1 infection in humans. We recently reported HIVD in the SBBC donor (subject D36), which coincided with a decrease in CD4 cell count and the presence of high viral load in both CSF and plasma, and was associated with compartmentalized evolution of the virus in CSF (Churchill *et al*, 2004). This study provided the first *in vivo* evidence that *nef*-deleted HIV-1 can invade the CNS of humans, and that *nef* was not essential for HIV-1 neuropathogenesis. The results further suggest other viral factors may compensate to increase the replication capacity of *nef*-deleted HIV-1 in the CNS to levels sufficient to cause neurologic disease.

Because viruses harbored by SBBC members contain alterations of NF-κB and Sp-1 binding sites in the LTR that may affect transcriptional activity and thus, replication capacity (Churchill *et al*, 2004, 2006; Deacon *et al*, 1995), we examined the nucleotide sequence and transcriptional activity of *nef*/LTR clones derived from blood and CSF of subject D36 to determine whether changes in LTR activity may contribute to CSF viral load and neuropathogenesis of *nef*-deleted HIV-1 infection. However, we acknowledge that without brain-biopsy tissue for comparison, it remains uncertain whether virus in the CSF is a true reflection of virus replicating in cells of the monocyte-macrophage lineage in the CNS. Peripheral blood mononuclear cell (PBMC)-derived HIV-1 isolated on six occasions prior to and during HIVD, and HIV-1 RNA purified from CSF taken on one occasion during HIVD was used in this study (Churchill *et al*, 2004). The collection of blood and CSF was in accordance with guidelines endorsed by the Australian Red Cross Blood Service human ethics committee. The viral phenotypes and analyses of V3 Env amino acid sequences have been described previously (Churchill *et al*, 2004). The clinical history of D36 has been described in detail previously (Churchill *et al*, 2004, 2006; Learmont *et al*, 1999). Briefly, in December 1998 the subject presented with a 3-month history of increasing forgetfulness, poor balance, action tremor, and urinary hesitancy. Clinical examination revealed that he was mentally slow and had bilateral extensor plantar responses and that tandem gait was mildly impaired. A magnetic resonance imaging scan of the subject's brain showed periventricular changes in the white matter. A neuropsychological assessment showed changes consistent with subcortical dementia. The clinical, laboratory, and neuropsychological results supported the diagnosis of moderate HIVD, which was the subject's AIDS defining illness. Clinical and laboratory data relevant to HIV-1 isolates used in the present study are summarized in Table 1. CD4+ T-cell counts decreased from 552 to 160 per μl between August 1995 and December 1998, which was accompanied by a steady increase in plasma HIV-1 RNA levels from 1100 to 15,183 copies/ml. The diagnosis of HIVD occurred with a CSF viral load of >750,000 HIV-1 RNA copies/ml. The subject was placed on a HAART regimen of abacavir, nevirapine, and zidovidine in January 1999, which suppressed plasma and CSF viral loads to below detectable levels and resolved the symptoms of HIVD (Churchill *et al*, 2004; Crowe *et al*, 2005).

The *nef*/LTR region of the HIV-1 genome was amplified either from phytohemagglutinin (PHA)-activated, interleukin-2–stimulated PBMCs that were infected with each of the blood derived HIV-1 isolates or from CSF HIV-1 RNA by reverse transcriptase–polymerase chain reaction (RT-PCR) using high-fidelity DNA polymerase and nested primers, as described elsewhere (Deacon *et al*, 1995; Rhodes *et al*, 2000). The products of six independent PCR reactions from each blood or CSF sample (corresponding

Table 1 Viruses, laboratory studies, and clinical history

Virus	*Date of blood/CSF sample*	*Years since infection*	*CD4+ T-cell count (cells/μl)*	*Plasma viral load (RNA copies/ml)*	*CSF viral load (RNA copies/ml)*	*Clinical history*
D36II	8/1995	14.4	552	1,100	N/A	Diagnosed with HIVD 12/1998.
D36V	7/1996	15.6	414	2,600	N/A	ABC, AZT, NVP
D36VIII	5/1997	16.4	540	4,020	N/A	(1/1999–9/2004)
D36IX	12/1997	17.0	390	7,500	N/A	ABC, NVP, 3TC
D36X	6/1998	17.5	325	15,183	N/A	(9/2004–present)
D36XI	12/1998	18.1	160	9,900	>750,000	

Note. CD4+ T-cell levels were measured by flow cytometry. HIV-1 RNA levels were measured using COBAS AMPLICOR HIV-1 monitor version 1.0 (Roche Molecular Diagnostic Systems, Branchburg, NJ) prior to 1999 and version 1.5 after 1999. HIV-1 RNA levels of <400 copies/ml (version 1.0) or <50 copies/ml (version 1.5) were considered below detection. BD, below detection; N/A, not available; HIVD, HIV-associated dementia; ABC, abacavir; AZT, zidovudine; NVP, nevirapine; 3TC, lamivudine.

to nucleotides 8671 to 9641 of HIV-1 NL4-3) were pooled and cloned into pGEM-T-easy (Promega, Madison, WI). Nucleotide sequences deduced from multiple, independent clones showed deletion mutations of varying magnitude within the *nef*-alone and *nef*/LTR overlap region as well as duplications and/or rearrangements of NF-κB and Sp-1 binding sites in the LTR (Churchill *et al*, 2004). These data are represented schematically in Figure 1. To determine transcriptional activity, three independent *nef*/LTR clones from each blood or CSF sample were subcloned into the pGL3 Basic plasmid (Promega), upstream of a *Firefly* luciferase reporter gene. The basal promoter activity of the *nef*/LTRs was examined by measuring *Firefly* luciferase activity in lysates of jurkat cells that were transfected by electroporation with 15 μg of each plasmid, at 72 h post transfection. The percentage of transfected jurkat cells was typically 40% to 50%, as judged by flourescence-activated cell sorting (FACS) analysis of cells transfected with pEGFP (data not shown). To control for transfection efficiency, cells were cotransfected with 0.5 μg of pGL3 Renilla plasmid (Promega), which contains a *Renilla* luciferase reporter gene under the control of a cytomegalovirus (CMV) promoter. Because *Renilla* and *Firefly* luciferase proteins have different substrate requirements, the activity of both proteins was measured using a dual-luciferase assay system (Promega), and *Firefly* luciferase activity measurements were normalized to *Renilla* luciferase activity.

When compared to basal transcriptional activity measured from the wild-type *nef*/LTR sequence of HIV-1 NL4-3, *nef*/LTRs of the sequential blood-derived D36 isolates exhibited modest (up to two-fold) increases in transcriptional activity, which, with the exception of *nef*/LTR of D36IX, reached statistical significance (Figure 1). In contrast, the *nef*/LTR of D36IX had transcriptional activity that was similar to that of NL4-3. Interestingly, the transcriptional activity of *nef*/LTRs cloned from CSF of D36 during HIVD was not only significantly greater than that of NL4-3 and other brain-derived wild-type control *nef*/LTRs cloned from YU-2 and JR-FL strains, but was significantly greater than that of the blood-derived *nef*/LTR clones. Similar results were obtained using 293T and CEM cells (data not shown). These results suggest the basal promoter activity of *nef*/LTRs cloned from CSF and most of the blood-derived isolates is augmented when compared with that of wild-type *nef*/LTR sequences of NL4-3, YU-2, and JR-FL.

To determine the relationship between basal transcriptional activity of *nef*/LTRs and changes in *nef*/LTR nucleotide sequence, a comparison was made between the *nef*/LTR structure and the corresponding basal transcription data (Figure 1). The increased basal transcriptional activity of *nef*/LTR clones from D36II, D36V, and D36VIII viruses was associated with the gain of two additional NF-κB binding sites and an additional Sp-1 binding site, and the presence of a fragmented 5′ nucleotide sequence in the *nef*-alone and *nef*/LTR overlap regions consisting of multiple deletion mutations. The loss of augmented basal transcriptional activity exhibited by *nef*/LTR of D36IX was associated with retention of most of the fragmented nucleotide sequence, but with the loss of one of the additional NF-κB binding sites and the additional Sp-1 binding site, suggesting that, in the context of a fragmented *nef* coding sequence, these two sites may be important for the enhanced level of transcription. However, augmented basal transcriptional activity was restored in D36X and D36XI by the removal of additional *nef* sequence, creating a single, large deletion spanning the *nef*-alone and the *nef*/LTR overlap regions. This suggests that removal of redundant *nef* sequence may compensate for the NF-κB and Sp-1 binding site losses in D36IX by forming a *nef*/LTR regulatory unit with greater efficiency. Of note, the *nef*/LTR of D36XI, which was isolated from blood during HIVD, regained the second additional NF-κB binding site. Thus, in the context of *nef*-deleted HIV-1 harbored by D36, duplication of NF-κB and Sp-1 binding sites or the removal of redundant *nef*-coding sequence are features that appear to be separately advantageous for enhancing basal transcription. Our results are consistent with those of previous studies that showed

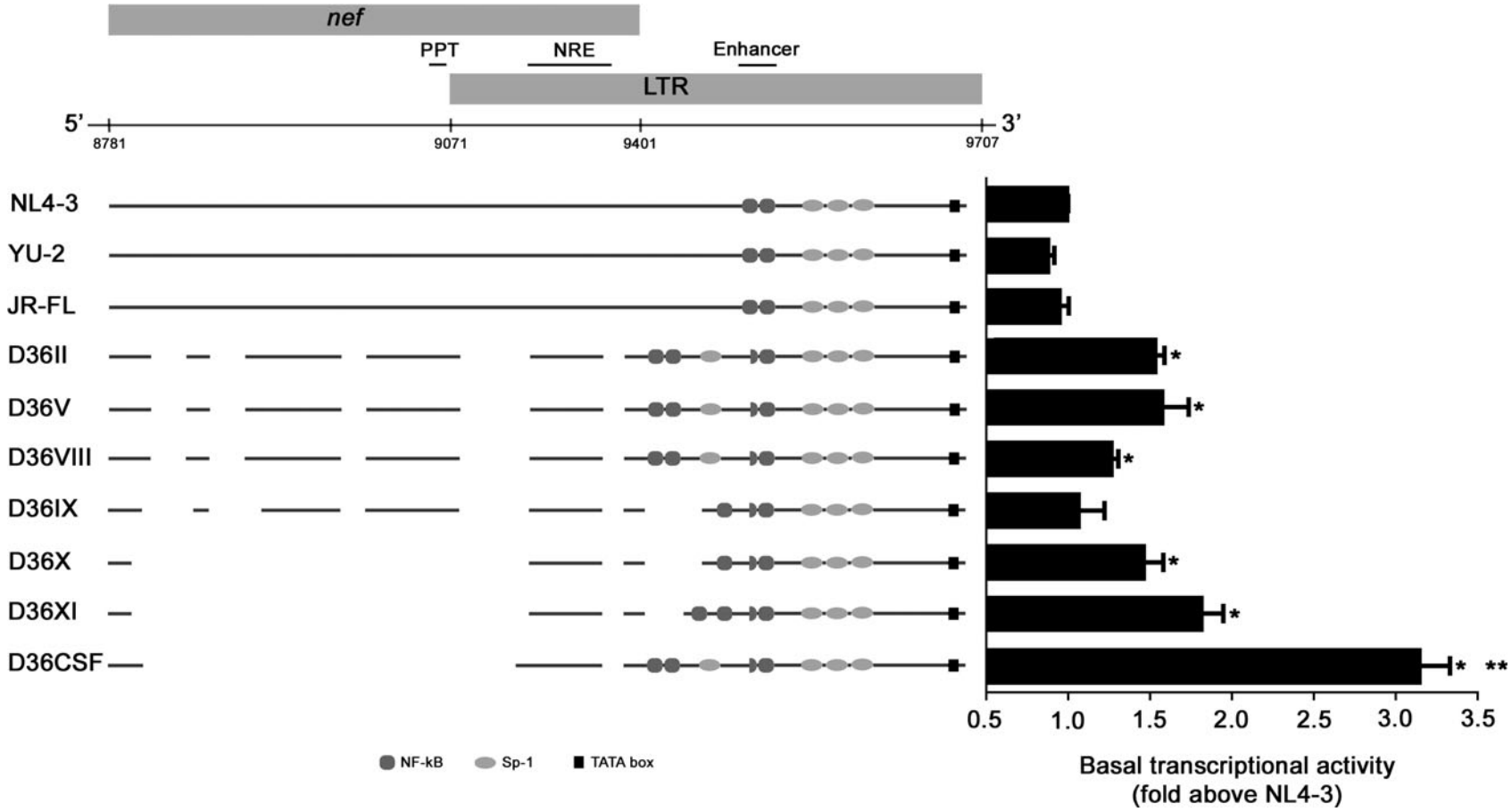

Figure 1 Basal transcriptional activity of blood- and CSF-derived *nef*/LTRs. A schematic of the *nef*/LTR structure depicting nucleotide sequence deletions and transcription factor binding sites is shown on the left, and represents the consensus of multiple clones sequenced (Churchill *et al*, 2004). Sequence representations are compared to that of wild-type HIV-1 (NL4-3). *Nef*/LTR sequences derived from the brain-derived YU-2 (Li *et al*, 1991, 1992) and JR-FL strains of HIV-1 (O'Brien *et al*, 1990) were included as additional wild-type controls. The solid lines indicate intact sequence and gaps indicate deletions. The basal transcriptional activity of three representative *nef*/LTR clones from each virus is shown on the right. The results represent the mean values obtained from three independent experiments. Error bars represent standard deviations. PPT, polypurine tract; NRE, negative regulatory element; LTR, long-terminal repeat. $^{*}P < .05$ for comparisons of transcriptional activity between D36 and NL4-3 *nef*/LTRs. $^{**}P < .05$ for comparisons of transcriptional activity between D36 CSF *nef*/LTRs, and blood-derived D36 and NL4-3 *nef*/LTRs. *P* values were calculated using a paired Student's *t* test.

enhanced basal transcriptional activity of subtype C LTR variants due to the presence of an additional NF-κB binding site (Jeeninga *et al*, 2000). The data also reflect the continually evolving nature of the *nef*/LTR in HIV-1 harbored by D36 that selects for strains with enhanced basal transcriptional activity, which may contribute to enhancing replication capacity of *nef*-deleted viruses.

The enhanced basal transcriptional activity of *nef*/LTRs cloned from CSF was associated with *nef*/LTR sequence deletions that were similar to those harbored by the "late emerging" D36X and D36XI viruses (Figure 1). However, the *nef*/LTRs derived from CSF also contained an arrangement of additional NF-κB and Sp-1 binding sites that was similar to the "earlier" D36II, D36V, and D36VIII viruses. Therefore, HIV-1 present in CSF of D36 harbored both *nef*/LTR features that appear to be separately advantageous for enhancement of basal transcription. This suggests a cooperative effect of changes in *nef* and LTR sequence may contribute to maximizing transcriptional activity of the CSF-derived *nef*/LTRs.

Infected cells producing high levels of HIV-1 in the CSF of D36 are likely to be activated. Furthermore, transcriptional activation of the LTR *in vivo* occurs in response to HIV-1 Tat. Therefore, we next measured the transcriptional activity of blood- and CSF-derived *nef*/LTRs in transfected jurkat cells under PMA- or Tat-activated conditions. Activation of control *nef*/LTRs derived from wild type NL4-3, YU-2 and JR-FL HIV-1 strains with PMA or Tat led to an approximately 6- and 58-fold increase in transcriptional activity, respectively, when compared to basal transcriptional activity (data not shown). When compared to transcriptional activity measured from wild-type control *nef*/LTRs, *nef*/LTRs of the sequential blood-derived D36 isolates exhibited modest (up to 1.5-fold) increases in transcriptional activity in response to PMA or Tat (Figure 2), which were similar to the modest increases in transcriptional activity measured under basal conditions (Figure 1). However, transcriptional activity of CSF-derived *nef*/LTRs in response to PMA or Tat was approximately 4.5-fold greater than that of wild-type control *nef*/LTRs, and 3- to 4.5-fold greater than that of the blood-derived D36 *nef*/LTR clones. These results demonstrate augmented promoter activity of *nef*/LTRs cloned from CSF of D36 during HIVD, under PMA- and Tat-activated conditions. Together, the results of basal, PMA- and Tat-activated

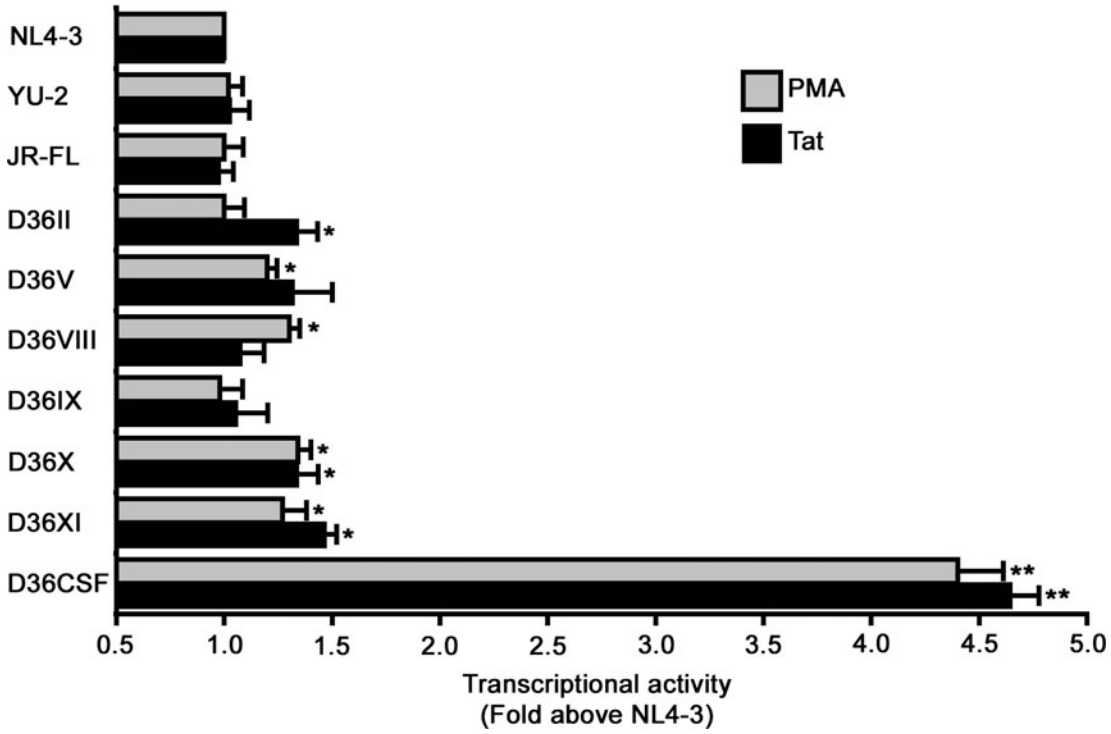

Figure 2 PMA- and Tat-activated transcriptional activity of blood- and CSF-derived nef/LTRs. Jurkat cells were cotransfected with 15 μg pGL3 Basic plasmid encoding *nef*/LTRs, 0.5 μg pGL3 Renilla plasmid, and 1.5 μl pTat72 plasmid, or were treated with 25 ng/ml PMA 24 h after being cotransfected with pGL3 Basic and pGL3 Renilla plasmids. Transcriptional activity of *nef*/LTRs under PMA or Tat activation was measured by luciferase assays as described in the text, and normalized to transcriptional activity measured from *nef*/LTR derived from HIV-1 NL4-3. *Nef*/LTR sequences derived from the brain-derived YU-2 (Li *et al*, 1991, 1992) and JR-FL strains of HIV-1 (O'Brien *et al*, 1990) were included as additional wild-type controls. The results represent the mean values obtained from three independent experiments. Error bars represent standard deviations. $^{*}P < .05$ for comparisons of transcriptional activity between D36 and NL4-3 *nef*/LTRs. $^{**}P < .05$ for comparisons of transcriptional activity between D36 CSF *nef*/LTRs, and blood-derived D36 and NL4-3 *nef*/LTRs. *P* values were calculated using a paired Student's *t* test.

transcriptional activation studies suggest that augmented transcriptional activity of CSF-derived clones may contribute to increasing the replication capacity of *nef*-deleted HIV-1 to levels that reflect the high viral load in CSF (Table 1), and which may be sufficient for *nef*-deleted HIV-1 to cause neurologic disease.

Our results are consistent with those of previous studies suggesting augmented LTR activity contributes to the neuropathogenesis of HIV-1 infection (reviewed in van Marle and Power, 2005). Changes in CCAAT/enhancer binding protein (C/EBP) binding sites, which have been linked to efficient HIV-1 replication in macrophages (Ross *et al*, 2001b), have been shown to occur in brain-derived LTRs from patients with HIVD, and affect LTR activity (Burdo *et al*, 2004a, 2004b; Hogan *et al*, 2003; Millhouse *et al*, 1998; Ross *et al*, 2001a; Schwartz *et al*, 2000). In addition, the LTR promoter proximal Sp1 binding site has been shown to interact with the Sp1 transcription factor family of transcription regulators, including both activators and repressors of promoter activity. Single-base mutations within this binding motif in the brain-derived, macrophage-tropic YU-2 strain of HIV-1 altered binding to this site, and resulted in a 40% loss of promoter activity when analyzed in both T-cell and astrocytic-cell lines (Millhouse *et al*, 1998). These studies suggest unique regulatory mechanisms governing HIV-1 replication are likely to be present in brain and may contribute to HIV-1 neuropathogenesis. However, more detailed studies using expanded numbers of brain-derived LTRs are required to fully elucidate the role of C/EBP and Sp1 motifs or other *cis*-acting elements in the transcriptional regulation of HIV-1 replication in brain. Of note, C/EBP binding sites occur in regions of the LTR that are deleted from D36 viruses (data not shown), whereas the Sp1 binding sites are maintained as high-affinity binding sites in both wild type and D36 variants. This suggests that other *cis*-acting regulatory elements may contribute to the augmented transcriptional activity and neuropathogenesis of *nef*/LTR-deleted HIV-1 harbored by D36.

Previous studies showed that LTR variants with augmented transcriptional activity enhance replication of HIV-1 (Jeeninga *et al*, 2000). To determine whether D36 *nef*/LTRs affect replication capacity of HIV-1 *in vitro*, full-length chimeric molecular clones of HIV-1 NL4-3 were constructed by replacing the wild-type, *nef*/LTR region (corresponding to nucleotides 8671 to 9641) with that of D36II-, D36IX-, D36XI-, and CSF-derived *nef*/LTRs (referred to as NL-D36II, NL-D36IX, NL-D36XI, and NL-D36CSF viruses, respectively). Virus stocks were produced by transfection of 293T cells, filtered through 0.45-μm filters, quantified by reverse transcriptase (RT) assays (Gorry *et al*, 1998) and stored at -80°C. Control viruses included wild-type NL4-3 and an isogenic strain containing a 222-bp deletion in the *nef* alone region surrounding the *Xho*I restriction site that does not overlap the LTR (NL4-3deltaNef) (Gorry *et al*, 1998). We examined the capacity of chimeric NL4-3 viruses carrying D36 *nef*/LTRs to replicate in PHA-activated PBMCs (PHA-PBMCs) compared to wild-type NL4-3 and the NL4-3deltaNef deletion mutant, as described previously (Gorry *et al*, 2001). Wild-type NL4-3 replicated rapidly to high levels, with peak virus replication occurring at day 3 post infection (Figure 3). In contrast, NL4-3deltaNef had reduced and delayed replication kinetics when compared to NL4-3, indicating that the presence of a full-length *nef* gene is required for optimal replication of NL4-3 in PHA-PBMCs. However, the differences in replication kinetics between wild type and *nef*-deleted NL4-3 were modest, consistent with previous studies (Miller *et al*, 1994; Ryan-Graham and Peden, 1995). Chimeric viruses containing blood-derived *nef*/LTRs also had delayed replication kinetics similar to NL4-3deltaNef, but the peak levels of replication that occurred at day 7 post infection differed; NL-D36II virus reached levels of replication that were similar to NL4-3deltaNef. NL-D36IX virus replicated to peak levels that were lower than NL4-3deltaNef, and NL-D36XI virus replicated to peak levels that were greater than NL4-3deltaNef. Thus, our results show a direct association between transcriptional activity of the blood-derived *nef*/LTRs (Figures 1 and 2), and the peak levels of virus replication achieved in PHA-PBMCs by chimeric viruses carrying the respective *nef*/LTRs. However, despite the *nef*/LTRs of D36II and D36XI having augmented basal and Tat-activated transcriptional activities compared to wild-type NL4-3 (Figures 1 and 2), augmented transcriptional activity of these clones was not sufficient to increase replication capacity of HIV-1 in PHA-PBMCs to levels achieved by NL4-3.

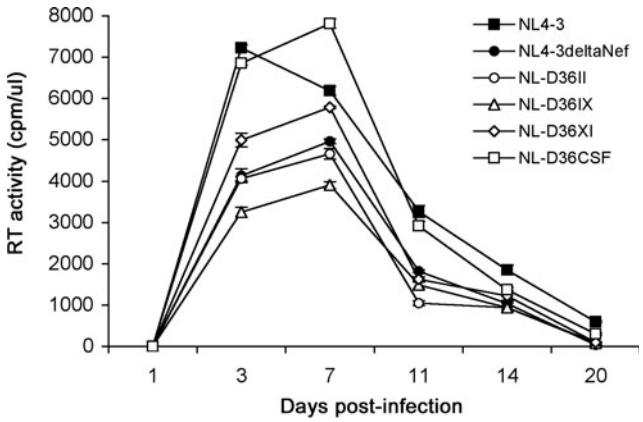

Figure 3 Replication of chimeric HIV-1 viruses in PHA-activated PBMCs. PHA-PBMC were infected with equivalent amounts of each chimeric or control virus, as described previously (Gorry *et al*, 2001; Gray *et al*, 2005), and cultured for 20 days. Virus production in culture supernatants was measured by RT assays. Values shown are means from triplicate infections. Error bars represent standard deviations. Results are representative of two independent experiments using cells from different donors.

In contrast to viruses carrying blood-derived *nef*/LTRs, NL-D36CSF virus replicated rapidly to high levels in PHA-PBMCs (Figure 3). The replication kinetics and levels of virus replication were similar to wild-type NL4-3 at day 3 post infection. However, NL-D36CSF continued to replicate to levels that exceeded those achieved by NL4-3, peaking at day 7 post infection. Thus, the *nef*/LTR derived from CSF of D36, which had augmented basal, PMA-, and Tat-activated transcriptional activities compared to wild-type and blood-derived D36 *nef*/LTRs (Figures 1 and 2), was sufficient to restore replication of HIV-1 in PHA-PBMCs to levels equal to or greater than those achieved by NL4-3. Together, our results suggest that unique structural features of the CSF-derived *nef*/LTR restore efficient replication capacity of *nef*-deleted HIV-1 in PHA-PBMCs by enhancing transcription.

Whether increased transcriptional activity of the CSF-derived *nef*/LTR actually caused or contributed to high levels of HIV-1 in CSF, or indeed to neuropathogenesis, is unclear. However, our results suggest that the unique structure of the CSF-derived *nef*/LTR may contribute to enhancing replication of *nef*-deleted HIV-1 in the CNS to levels sufficient to cause neurologic disease. This possibility is supported by previous studies that showed LTR variants with additional NF-κB binding sites are hyperresponsive to activation by tumor necrosis factor (TNF)-α (Jeeninga *et al*, 2000), which is a proinflammatory cytokine elevated in HIVD and implicated in HIV-1 neuropathogenesis (Wesselingh *et al*, 1993). However, high levels of viral antigen in the CNS *per se* is not sufficient to cause neurologic disease (Joag *et al*, 1995; Korber *et al*, 1994; Mankowski *et al*, 1997; Power *et al*, 1994), suggesting that additional viral factors other than those that enhance replication in brain are necessary for HIV-1 neurovirulence. To this end, it is important to note that the Env of blood-derived D36 viruses is dual-tropic (R5X4), whereas the Env of HIV-1 in CSF of D36 is CCR5-restricted (R5) (Churchill *et al*, 2004). Although R5X4 strains have been recovered from brain (Gorry *et al*, 2001, 2002; Ohagen *et al*, 2003; Peters *et al*, 2004), their presence is considered to be infrequent compared to R5 strains, which cause neurologic disease in the vast majority of subjects (Albright *et al*, 1999; He *et al*, 1997; Li *et al*, 1999; Shieh *et al*, 1998; Smit *et al*, 2001). In addition, when compared to brain-derived R5X4 viruses, blood-derived D36 viruses lack the ability to replicate efficiently in monocyte-derived macrophages (data not shown). Because macrophage (M)-tropism of R5 and R5X4 HIV-1 strains is necessary for neurotropism (Gorry *et al*, 2001; Peters *et al*, 2004), the R5X4 Envs of blood-derived D36 viruses are not likely to be neurotropic. Further studies are required to determine whether the R5 Env of the CSF-derived HIV-1 isolate exhibits M-tropism and neurotropism. However, it is possible that enhanced transcriptional activity of the CSF-derived *nef*/LTR, coupled with a likely M-tropic R5 Env, may be sufficient for neurotropism and neurovirulence of *nef*-deleted HIV-1 harbored by D36.

The molecular mechanisms underlying the neuropathogenicity of the CSF-derived virus remain to be determined, but possible explanations include (1) increased CCR5 affinity and/or reduced CD4/CCR5 dependence of the Env, which has been observed for other neurovirulent R5 HIV-1 strains (Gorry *et al*, 2002; Martin-Garcia *et al*, 2005, 2006; Peters *et al*, 2004; Shieh *et al*, 2000), and (2) increased Env-mediated fusion, which has been shown to increase the pathogenicity of *nef*-deleted SIV (Alexander *et al*, 2003). Further studies are required to elucidate the role of Env, and the interplay between Env function and *nef*/LTR in the neuropathogenesis of *nef*-deleted HIV-1 harbored by D36.

In conclusion, the present study demonstrated unique *nef*/LTR sequences in the CSF of a long-term survivor infected with *nef*-deleted HIV-1 who developed HIVD. Augmented basal, PMA-, and Tat-activated transcriptional activities of the CSF-derived *nef*/LTR were associated with high viral load in the CSF and enhanced HIV-1 replication capacity. Our results indicate that *nef*-deleted HIV-1 may undergo compartmentalized evolution in the CNS and cause neurologic disease in humans, and suggest that changes in *nef*/LTR may to contribute to neurovirulence of *nef*-deleted HIV-1 variants.

References

Albright AV, Shieh JT, Itoh T, Lee B, Pleasure D, O'Connor MJ, Doms RW, Gonzalez-Scarano F (1999). Microglia express CCR5, CXCR4, and CCR3, but of these, CCR5 is the principal coreceptor for human immunodeficiency virus type 1 dementia isolates. *J Virol* **73:** 205–213.

Alexander L, Illyinskii PO, Lang SM, Means RE, Lifson J, Mansfield K, Desrosiers RC (2003). Determinants of increased replicative capacity of serially passaged simian immunodeficiency virus with nef deleted in rhesus monkeys. *J Virol* **77:** 6823–6835.

Burdo TH, Gartner S, Mauger D, Wigdahl B (2004a). Region-specific distribution of human immunodeficiency virus type 1 long terminal repeats containing specific configurations of CCAAT/enhancer-binding protein site II in brains derived from demented and nondemented patients. *J NeuroVirol* **10(Suppl 1):** 7–14.

Burdo TH, Nonnemacher M, Irish BP, Choi CH, Krebs FC, Gartner S, Wigdahl B (2004b). High-affinity interaction between HIV-1 Vpr and specific sequences that span the C/EBP and adjacent NF-kappaB sites within the HIV-1 LTR correlate with HIV-1-associated dementia. *DNA Cell Biol* **23:** 261–269.

Chakrabarti L, Baptiste V, Khatissian E, Cumont MC, Aubertin AM, Montagnier L, Hurtrel B (1995). Limited viral spread and rapid immune response in lymph nodes of macaques inoculated with attenuated

simian immunodeficiency virus. *Virology* **213:** 535–548.

Churchill MJ, Rhodes DI, Learmont JC, Sullivan JS, Wesselingh SL, Cooke IR, Deacon NJ, Gorry PR (2006). Longitudinal analysis of human immunodeficiency virus type 1 nef/long terminal repeat sequences in a cohort of long-term survivors infected from a single source. *J Virol* **80:** 1047–1052.

Churchill M, Sterjovski J, Gray L, Cowley D, Chatfield C, Learmont J, Sullivan JS, Crowe SM, Mills J, Brew BJ, Wesselingh SL, McPhee DA, Gorry PR (2004). Longitudinal analysis of nef/long terminal repeat-deleted HIV-1 in blood and cerebrospinal fluid of a long-term survivor who developed HIV-associated dementia. *J Infect Dis* **190:** 2181-2186.

Crowe SM, Ho DD, Marriott D, Brew B, Gorry PR, Sullivan JS, Learmont J, Mills J (2005). In vivo replication kinetics of a nef-deleted strain of HIV-1. *AIDS* **19:** 842–843.

Deacon NJ, Tsykin A, Solomon A, Smith K, Ludford-Menting M, Hooker DJ, McPhee DA, Greenway AL, Ellett A, Chatfield C, *et al* (1995). Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion donor and recipients. *Science* **270:** 988–991.

Desrosiers RC, Lifson JD, Gibbs JS, Czajak SC, Howe AY, Arthur LO, Johnson RP (1998). Identification of highly attenuated mutants of simian immunodeficiency virus. *J Virol* **72:** 1431–1437.

Dunfee R, Thomas E, Gorry PR, Wang J, Ancuta P, Gabuzda D (2006). Mechanisms of HIV-1 Neurotropism. *Curr HIV Res* **4:** 267–278.

Gabuzda D, Wang J (2000). Chemokine receptors and mechanisms of cell death in HIV neuropathogenesis. *J NeuroVirol* **6(Suppl 1):** S24–S32.

Gabuzda D, Wang J, Gorry PR (2002). HIV-1-associated dementia. In: *Universes in delicate balance: chemokines and the nervous system*. Ransohoff RM, Suzuki K, Proudfoot AEI, Hickey WF, and Harrison JK (eds). Amsterdam: Elsevier Science, pp 345–360.

Gonzalez-Scarano F, Martin-Garcia J (2005). The neuropathogenesis of AIDS. *Nat Rev Immunol* **5:** 69–81.

Gorry P, Purcell D, Howard J, McPhee D (1998). Restricted HIV-1 infection of human astrocytes: potential role of nef in the regulation of virus replication. *J NeuroVirol* **4:** 377–386.

Gorry PR, Bristol G, Zack JA, Ritola K, Swanstrom R, Birch CJ, Bell JE, Bannert N, Crawford K, Wang H, Schols D, De Clercq E, Kunstman K, Wolinsky SM, Gabuzda D (2001). Macrophage tropism of human immunodeficiency virus type 1 isolates from brain and lymphoid tissues predicts neurotropism independent of coreceptor specificity. *J Virol* **75:** 10073–10089.

Gorry PR, Taylor J, Holm GH, Mehle A, Morgan T, Cayabyab M, Farzan M, Wang H, Bell JE, Kunstman K, Moore JP, Wolinsky SM, Gabuzda D (2002). Increased CCR5 affinity and reduced CCR5/CD4 dependence of a neurovirulent primary human immunodeficiency virus type 1 isolate. *J Virol* **76:** 6277–6292.

Gray L, Sterjovski J, Churchill M, Ellery P, Nasr N, Lewin SR, Crowe SM, Wesselingh S, Cunningham AL, Gorry PR (2005). Uncoupling coreceptor usage of human immunodeficiency virus type 1 (HIV-1) from macrophage tropism reveals biological properties of CCR5-restricted HIV-1 isolates from patients with acquired immunodeficiency syndrome. *Virology* **337:** 384–398.

He J, Chen Y, Farzan M, Choe H, Ohagen A, Gartner S, Busciglio J, Yang X, Hofmann W, Newman W, Mackay CR, Sodroski J, Gabuzda D (1997). CCR3 and CCR5 are co-receptors for HIV-1 infection of microglia. *Nature* **385:** 645–649.

Hofmann-Lehmann R, Vlasak J, Williams AL, Chenine AL, McClure HM, Anderson DC, O'Neil S, Ruprecht RM (2003). Live attenuated, nef-deleted SIV is pathogenic in most adult macaques after prolonged observation. *AIDS* **17:** 157–166.

Hogan TH, Stauff DL, Krebs FC, Gartner S, Quiterio SJ, Wigdahl B (2003). Structural and functional evolution of human immunodeficiency virus type 1 long terminal repeat CCAAT/enhancer binding protein sites and their use as molecular markers for central nervous system disease progression. *J NeuroVirol* **9:** 55–68.

Iafrate AJ, Carl S, Bronson S, Stahl-Hennig C, Swigut T, Skowronski J, Kirchhoff F (2000). Disrupting surfaces of nef required for downregulation of CD4 and for enhancement of virion infectivity attenuates simian immunodeficiency virus replication in vivo. *J Virol* **74:** 9836–9844.

Jeeninga RE, Hoogenkamp M, Armand-Ugon M, de Baar M, Verhoef K, Berkhout B. (2000). Functional differences between the long terminal repeat transcriptional promoters of human immunodeficiency virus type 1 subtypes A through G. *J Virol* **74:** 3740–3751.

Joag SV, Stephens EB, Galbreath D, Zhu GW, Li Z, Foresman L, Zhao LJ, Pinson DM, Narayan O (1995). Simian immunodeficiency virus SIVmac chimeric virus whose env gene was derived from SIV-encephalitic brain is macrophage-tropic but not neurovirulent. *J Virol* **69:** 1367–1369.

Kestler HW 3rd, Ringler DJ, Mori K, Panicali DL, Sehgal PK, Daniel MD, Desrosiers RC (1991). Importance of the nef gene for maintenance of high virus loads and for development of AIDS. *Cell* **65:** 651–662.

Kirchhoff F, Greenough TC, Brettler DB, Sullivan JL, Desrosiers RC (1995). Brief report: absence of intact nef sequences in a long-term survivor with nonprogressive HIV-1 infection. *N Engl J Med* **332:** 228–232.

Kondo M, Shima T, Nishizawa M, Sudo K, Iwamuro S, Okabe T, Takebe Y, Imai M (2005). Identification of attenuatedvariants of HIV-1 circulating recombinant form 01_AE that are associated with slow disease progression due to gross genetic alterations in the nef/long terminal repeat sequences. *J Infect Dis* **192:** 56–61.

Korber BT, MacInnes K, Smith RF, Myers G (1994). Mutational trends in V3 loop protein sequences observed in different genetic lineages of human immunodeficiency virus type 1. *J Virol* **68:** 6730–6744.

Learmont J, Tindall B, Evans L, Cunningham A, Cunningham P, Wells J, Penny R, Kaldor J, Cooper DA (1992). Long-term symptomless HIV-1 infection in recipients of blood products from a single donor. *Lancet* **340:** 863–867.

Learmont JC, Geczy AF, Mills J, Ashton LJ, Raynes-Greenow CH, Garsia RJ, Dyer WB, McIntyre L, Oelrichs RB, Rhodes DI, Deacon NJ, Sullivan JS (1999). Immunologic and virologic status after 14 to 18 years of infection with an attenuated strain of HIV-1. A report from the Sydney Blood Bank Cohort. *N Engl J Med* **340:** 1715–1722.

Li S, Juarez J, Alali M, Dwyer D, Collman R, Cunningham A, Naif HM (1999). Persistent CCR5 utilization and enhanced macrophage tropism by primary blood human immunodeficiency virus type 1 isolates from advanced stages of disease and comparison to tissue-derived isolates. *J Virol* **73:** 9741–9755.

Li Y, Hui H, Burgess CJ, Price RW, Sharp PM, Hahn BH, Shaw GM (1992). Complete nucleotide sequence, genome organization, and biological properties of human immunodeficiency virus type 1 in vivo: evidence for limited defectiveness and complementation. *J Virol* **66:** 6587-6600.

Li Y, Kappes JC, Conway JA, Price RW, Shaw GM, Hahn BH (1991). Molecular characterization of human immunodeficiency virus type 1 cloned directly from uncultured human brain tissue: identification of replication-competent and -defective viral genomes. *J Virol* **65:** 3973–3985.

Mankowski JL, Flaherty MT, Spelman JP, Hauer DA, Didier PJ, Amedee AM, Murphey-Corb M, Kirstein LM, Munoz A, Clements JE, Zink MC (1997). Pathogenesis of simian immunodeficiency virus encephalitis: viral determinants of neurovirulence. *J Virol* **71:** 6055–6060.

Mariani R, Kirchhoff F, Greenough TC, Sullivan JL, Desrosiers RC, Skowronski J (1996). High frequency of defective nef alleles in a long-term survivor with non-progressive human immunodeficiency virus type 1 infection. *J Virol* **70:** 7752–7764.

Martin-Garcia J, Cao W, Varela-Rohena A, Plassmeyer ML, Gonzalez-Scarano F (2006). HIV-1 tropism for the central nervous system: brain-derived envelope glycoproteins with lower CD4 dependence and reduced sensitivity to a fusion inhibitor. *Virology* **346:** 169–179.

Martin-Garcia J, Cocklin S, Chaiken IM, Gonzalez-Scarano F (2005). Interaction with CD4 and antibodies to CD4-induced epitopes of the envelope gp120 from a microglial cell-adapted human immunodeficiency virus type 1 isolate. *J Virol* **79:** 6703–6713.

Messmer D, Ignatius R, Santisteban C, Steinman RM, Pope M (2000). The decreased replicative capacity of simian immunodeficiency virus SIVmac239Delta(nef) is manifest in cultures of immature dendritic cellsand T cells. *J Virol* **74:** 2406-2413.

Miller MD, Warmerdam MT, Gaston I, Greene WC, Feinberg MB (1994). The human immunodeficiency virus-1 nef gene product: a positive factor for viral infection and replication in primary lymphocytes and macrophages. *J Exp Med* **179:** 101–113.

Millhouse S, Krebs FC, Yao J, McAllister JJ, Conner J, Ross H, Wigdahl B (1998). Sp1 and related factors fail to interact with the NF-kappaB-proximal G/C box in the LTR of a replication competent, brain-derived strain of HIV-1 (YU-2). *J NeuroVirol* **4:** 312–323.

O'Brien WA, Koyanagi Y, Namazie A, Zhao JQ, Diagne A, Idler K, Zack JA, Chen IS (1990). HIV-1 tropism for mononuclear phagocytes can be determined by regions of gp120 outside the CD4-binding domain. *Nature* **348:** 69–73.

Ohagen A, Devitt A, Kunstman KJ, Gorry PR, Rose PP, Korber B, Taylor J, Levy R, Murphy RL, Wolinsky SM, Gabuzda D (2003). Genetic and functional analysis of full-length human immunodeficiency virus type 1 env genes derived from brain and blood of patients with AIDS. *J Virol* **77:** 12336–12345.

Peters PJ, Bhattacharya J, Hibbitts S, Dittmar MT, Simmons G, Bell J, Simmonds P, Clapham PR (2004). Biological analysis of human immunodeficiency virus type 1 R5 envelopes amplified from brain and lymph node tissues of AIDS patients with neuropathology reveals two distinct tropism phenotypes and identifies envelopes in the brain that confer an enhanced tropism and fusigenicity for macrophages. *J Virol* **78:** 6915–6926.

Power C, McArthur JC, Johnson RT, Griffin DE, Glass JD, Perryman S, Chesebro B (1994). Demented and non-demented patients with AIDS differ in brain-derived human immunodeficiency virus type 1 envelope sequences. *J Virol* **68:** 4643–4649.

Rhodes DI, Ashton L, Solomon A, Carr A, Cooper D, Kaldor J, Deacon N (2000). Characterization of three nef-defective human immunodeficiency virus type 1 strains associated with long-term nonprogression. Australian Long-Term Nonprogressor Study Group. *J Virol* **74:** 10581–10588.

Ross HL, Gartner S, McArthur JC, Corboy JR, McAllister JJ, Millhouse S, Wigdahl B (2001a). HIV-1 LTR C/EBP binding site sequence configurations preferentially encountered in brain lead to enhanced C/EBP factor binding and increased LTR-specific activity. *J NeuroVirol* **7:** 235–249.

Ross HL, Nonnemacher MR, Hogan TH, Quiterio SJ, Henderson A, McAllister JJ, Krebs FC, Wigdahl B (2001b). Interaction between CCAAT/enhancer binding protein and cyclic AMP response element binding protein 1 regulates human immunodeficiency virus type 1 transcription in cells of the monocyte/macrophage lineage. *J Virol* **75:** 1842–1856.

Ryan-Graham MA, Peden KW (1995). Both virus and host components are important for the manifestation of a Nef- phenotype in HIV-1 and HIV-2. *Virology* **213:** 158–168.

Sacktor N, McDermott MP, Marder K, Schifitto G, Selnes OA, McArthur JC, Stern Y, Albert S, Palumbo D, Kieburtz K, De Marcaida JA, Cohen B, Epstein L (2002). HIV-associated cognitive impairment before and after the advent of combination therapy. *J NeuroVirol* **8:** 136–142.

Schwartz C, Catez P, Rohr O, Lecestre D, Aunis D, Schaeffer E (2000). Functional interactions between C/EBP, Sp1, and COUP-TF regulate human immunodeficiency virus type 1 gene transcription in human brain cells. *J Virol* **74:** 65–73.

Shieh JT, Albright AV, Sharron M, Gartner S, Strizki J, Doms RW, Gonzalez-Scarano F (1998). Chemokine receptor utilization by human immunodeficiency virus type 1 isolates that replicate in microglia. *J Virol* **72:** 4243–4249.

Shieh JT, Martin J, Baltuch G, Malim MH, Gonzalez-Scarano F (2000). Determinants of syncytium formation in microglia by human immunodeficiency virus type 1: role of the V1/V2 domains. *J Virol* **74:** 693–701.

Singh DK, McCormick C, Pacyniak E, Griffin D, Pinson DM, Sun F, Berman NE, Stephens EB (2002). Pathogenic and nef-interrupted simian-human immunodeficiency viruses traffic to the macaque CNS and cause astrocytosis early after inoculation. *Virology* **296:** 39–51.

Smit TK, Brew BJ, Tourtellotte W, Morgello S, Gelman BB, Saksena NK (2004). Independent evolution of human immunodeficiency virus (HIV) drug resistance mutations in diverse areas of the brain in HIV-infected patients, with and without dementia, on antiretroviral treatment. *J Virol* **78:** 10133–10148.

Smit TK, Wang B, Ng T, Osborne R, Brew B, Saksena NK (2001). Varied tropism of HIV-1 isolates derived from different regions of adult brain cortex discriminate between patients with and without AIDS dementia complex (ADC): evidence for neurotropic HIV variants. *Virology* **279:** 509–526.

Strain MC, Letendre S, Pillai SK, Russell T, Ignacio CC, Gunthard HF, Good B, Smith DM, Wolinsky SM, Furtado M, Marquie-Beck J, Durelle J, Grant I, Richman DD, Marcotte T, McCutchan JA, Ellis RJ, Wong JK (2005). Genetic composition of human immunodeficiency virus type 1 in cerebrospinal fluid and blood without treatment and during failing antiretroviral therapy. *J Virol* **79:** 1772–1788.

Thompson KA, Kent SJ, Gahan ME, Purcell DF, McLean CA, Preiss S, Dale CJ, Wesselingh SL (2003). Decreased neurotropism of nef long terminal repeat (nef/LTR)-deleted simian immunodeficiency virus. *J NeuroVirol* **9:** 442–451.

van Marle G, Power C (2005). Human immunodeficiency virus type 1 genetic diversity in the nervous system: evolutionary epiphenomenon or disease determinant? *J NeuroVirol* **11:** 107–128.

Wesselingh S, Power C, Glass JD, Tyor WR, McArthur JC, Farber JM, Griffin JW, Griffin DE (1993). Intracerebral cytokine messenger RNA expression in acquired immunodeficiency syndrome dementia. *Ann Neurol* **33:** 576–582.