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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

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Previous studies have shown that two CCAAT/enhancer binding protein (C/EBP) binding sites (sites I and II) within the human immunodeficiency virus type 1 (HIV-1) long terminal repeat (LTR) are critically important for efficient virus replication within cells of the monocyte lineage, a primary cell type infected by HIV-1. Sequence variation at C/EBP sites I and II has been shown to alter the affinity of C/EBP factors to these sites. Specifically, sequence variation within C/EBP binding site II has been shown to alter binding of purified C/EBP β protein and basal activity of the HIV-1 LTR. We have previously demonstrated that the C/EBP site II consensus cladeB (ConB) variant was highly conserved in brain- and peripheral blood–derived LTRs of individuals with advanced HIV-1 disease. Given these important observations, the regional distribution of LTRs containing the C/EBP site II ConB variant derived from brain tissues of patients with and without HIV-1–associated dementia (HIVD) was examined. A statistically significant difference was found in the distribution of LTRs containing the C/EBP site II ConB variant in brain regions derived from patients with and without HIVD. In addition, we have previously shown that LTRs containing C/EBP site II 4C and 6G variants (designated according to the position at which nucleotide change occurred relative to ConB, followed by the actual nucleotide found at the variant position) were only found in brain tissue of patients with HIVD. As an extension of these observations, the regional distribution of LTRs containing C/EBP site II 4C or 6G variants derived from the brains of patients with HIVD was examined and a statistically significant difference was observed. We have shown that LTRs containing a low-affinity C/EBP site II 4C variant accumulated in the cerebellum. LTRs containing the 4C site variant in conjunction with the consensus cladeB (ConB) site I exhibited the lowest basal LTR activity of any of the LTRs examined. These results suggest that LTRs containing the C/EBP site II 4C configuration may promote the establishment of a latent provirus in the cerebellum, a region of the HIVD brain that exhibits little viral gene expression. Furthermore, LTRs containing a high affinity C/EBP site II 6G variant accumulated

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in the mid-frontal gyrus, a site of highly productive replication. In addition, LTRs containing the C/EBP site II 6G variant with the ConB at site I exhibited the highest basal LTR activity. In conclusion, distinct LTR populations with specific C/EBP site II configurations were found in different neuroanatomical regions of the brain, potentially due to differences in the molecular architecture of the LTR, viral entry pathways, and/or brain microenvironments. *Journal of NeuroVirology* (2004) **10(suppl. 1),** 7–14.

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Introduction

Human immunodeficiency virus type 1 (HIV-1) infection of the central nervous system (CNS) occurs soon after initial infection (Davis *et al*, 1992; Palmer *et al*, 1994). However, under most circumstances, prolonged productive viral replication with the formation of multinucleated giant cells and other associated cytopathogenic effects are likely to occur only after severe immunosuppression, leading to HIV-1–associated dementia (HIVD). It has been demonstrated that levels of viral RNA in the cerebrospinal fluid (CSF) correlate with the presence of HIVD (Brew *et al*, 1997). Radiographic evaluation of the HIVD brain demonstrates general atrophy, narrowing of the gyri and widening of the sulci, and mild edema of the subcortical and deep structures (Zink *et al*, 1999). In addition, multinucleated giant cells, a pathological hallmark of HIVD, have been demonstrated in the cortex, but are more prevalently localized within the subcortical white matter, and basal ganglia (Zink *et al*, 1999). In parallel with these studies, the amount of HIV-1 proviral DNA in different brain regions has been quantified and the highest levels have been shown to be in the medial temporal lobe (including subcortical white matter and hippocampus), with slightly lower levels in the basal ganglia (Fujimura *et al*, 1997). More recently, an additional study has demonstrated that the highest level of HIV-1 RNA was detected in subcortical regions, the deep white matter, and caudate of brain tissue derived from patients with HIVD (McClernon *et al*, 2001). These studies have clearly suggested that brain pathology associated with HIVD is localized to specific regions within the brain.

HIV-1 long terminal repeat (LTR) sequence variation affects viral replication and changes in this regulatory element may ultimately impact disease progression. Studies have demonstrated that HIV-1 LTRs evolve in a compartmentalized manner (Krebs *et al*, 2001). One study of multiple LTR sequences derived from different tissues of a single HIV-1–infected individual indicated that LTRs derived from specific tissues may be classified as phylogenetically distinct variants (Ait-Khaled *et al*, 1995). Phylogenetic analyses of HIV-1 LTR quasispecies from lymph node, spleen, lung, dorsal root ganglion, spinal cord, and peripheral blood have indicated that the LTR sequence variants cluster according to their tissue origin (Ait-Khaled *et al*, 1995). Spinal cord and dorsal root ganglion variants retained prototypic nuclear factor (NF)-κB binding sites, but exhibited seven sequence alterations within the tax responsive element (TAR) sequence, which significantly reduced binding of the viral transactivator protein, Tat. In an earlier report, this group summarized similar phylogenetic analyses and demonstrated a distinct polarization between HIV-1 variants from peripheral blood and lymph nodes (Ait-Khaled and Emery, 1994).

A number of investigators have compared HIV-1 *env* gene sequences from viruses isolated from different regions of brain (Chang *et al*, 1998; Liu *et al*, 2000; Shapshak *et al*, 1999), or compared brain-derived sequences with those recovered from other tissues (Chang *et al*, 1998; Epstein *et al*, 1991; Gartner *et al*, 1997; Korber *et al*, 1994; Liu *et al*, 2000; Power *et al*, 1994; Reddy *et al*, 1996). Some have also attempted to identify specific *env* motifs associated with HIVD (Power *et al*, 1994) or neurovirulence (Chang *et al*, 1998; Power *et al*, 1994; Shapshak *et al*, 1999). Many of these studies have focused exclusively on the hypervariable 3 (V3) loop of the gp120 region of *env* because this region contains determinants associated with macrophage tropism, although some evidence suggests that hypervariable region 4 (V4) can better distinguish brain-derived HIV-1 species from those in lymphocytes (Gartner *et al*, 1997). Chang and colleagues (1998) examined V3 sequences recovered from six different regions of brain collected from a patient who died with HIVD. They found genetically distinct V3 sequences within different brain regions, as well as unique V3 sequences within the frontal cortex, a region that also harbored multinucleated giant cells, an indicator of active viral replication. Power and colleagues (1994) sequenced the V3 region from brain and spleen tissues from 22 patients, and identified two amino acid positions (one in a sequence flanking the V3 region) associated with the presence of HIVD. In their study of multiple regions of brain from three patients (two with demonstrated HIVD and histology-proven encephalitis) Shapshak and coworkers (1999) observed independent region-specific differences within the V1–V5 region of gp120, and also identified significant amino acid signature patterns associated with particular neuroanatomical regions. Although the number of cases evaluated in each of these studies was limited, considered together, they suggest that HIV-1 evolution can proceed independently within the brain.

CCAAT/enhancer binding protein (C/EBP) binding sites I and II within the viral LTR have been shown to be critically important for efficient HIV-1 replication within cells of the monocyte/macrophage lineage, a primary target of HIV-1 infection in the brain (Henderson *et al*, 1995, 1996; Hogan *et al*, 2002; Ross *et al*, 2001a, 2001b). Studies demonstrated that at least one C/EBP site was required for replication of HIV-1 in the U-937 monocytic cell line as well as in primary cells of the monocyte/macrophage lineage. However, these sites were dispensable for replication of HIV-1 in T-cell lines and primary T-cell populations (Henderson and Calame, 1997; Henderson *et al*, 1995, 1996). We have previously demonstrated that C/EBP site II was highly conserved in brain-derived HIV-1 LTR populations, with 88.6% of LTRs containing the C/EBP site II ConB variant (Ross *et al*, 2001a). The high degree of C/EBP site II conservation in brain-derived LTRs was likely important in LTR regulation because the C/EBP binding site II consensus cladeB (ConB) variant exhibits a relatively high affinity for members of the C/EBP transcription factor family (Ross *et al*, 2001a). In addition, we have demonstrated that LTRs containing C/EBP site II 4C and 6G sequence variants were only found in brain tissues derived from patients with HIVD (Hogan *et al*, 2003b) and not in brain tissues derived from patients without HIVD. The studies reported herein have focused on sequence variation within C/EBP site II of HIV-1 LTRs derived from six regions of the brain obtained from patients with and without HIVD. We have also examined the C/EBP site II variants with respect to their relative affinity for C/EBP factors and their impact on basal LTR activity in transient transfection analyses.

Results

Naturally occurring sequence variation at C/EBP site II in the HIV-1 LTR impacts C/EBP factor recruitment from nuclear extracts derived from monocytic U-937 cells

Previous studies have indicated that the C/EBP site II ConB variant was preferentially encountered in brain- and peripheral blood–derived LTRs (Hogan *et al*, 2003a, 2003b; Ross *et al*, 2001a). In addition, the LTRs containing C/EBP site II 4C and 6G variants were found only in brain tissues derived from patients with HIVD (Hogan *et al*, 2003b). The C/EBP site II variants were designated according to the position at which base pair change occurred relative to the clade B consensus sequence, followed by the actual base pair that was found at the variant position (Figure 1A). To determine the impact of binding site sequence variation on recruitment of C/EBP proteins, electrophoretic mobility shift (EMS) analysis was performed. Probes containing C/EBP site II ConB, 6G, or 4C variants were reacted with nuclear

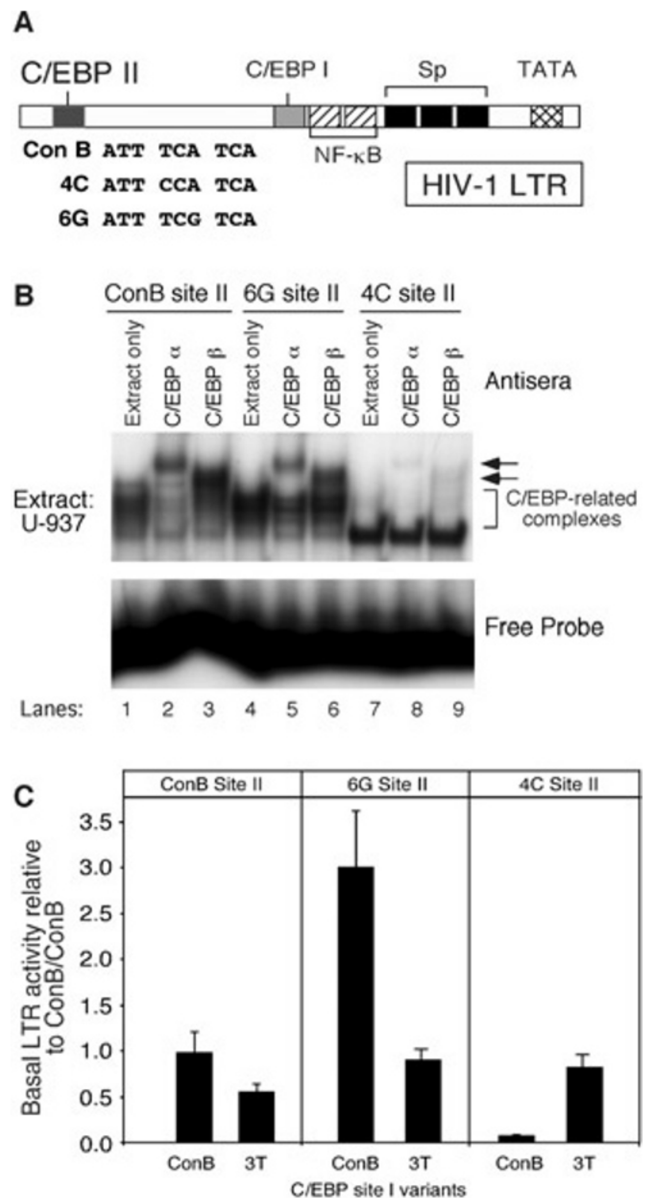

Figure 1 (**A**) Physical structure of the HIV-1 LTR and C/EBP site II variants. Depicted is a pictorial representation of the HIV-1 LTR with *cis*-acting elements, including C/EBP, NF-κB, Sp sites, and TATA box. C/EBP site II sequence variants ConB, 4C, and 6G are shown below the physical map. (**B**) C/EBP factors are present in DNA-protein complexes formed between monocytic nuclear extracts and the C/EBP site II ConB and 6G variants. U-937 nuclear extract was reacted with the C/EBP site II ConB, 6G, and 4C variants in EMS analyses. Brackets to the right of the panels indicate the DNA-protein complexes specific to C/EBP binding site. C/EBP-specific monoclonal antibodies were added to the indicated lanes. Arrows to the right of the panels indicate supershifted complexes. Free probe is shown below. (**C**) Impact of selected *cis*-acting C/EBP site variants on LTR activity. Transient expression analyses utilizing a dual luciferase reporter system (see Materials and methods) were employed to assess the effect of altering C/EBP binding sites I and II sequences on LTR function. Results comparing the basal activity of each construct in the U-937 monocytic cell line are presented relative to ConB/ConB (both C/EBP sites I and II contain the ConB configuration) basal LTR activity.

extracts prepared from the monocytic U-937 cell line (Figure 1B). As indicated in Figure 1B, DNA-protein complex formation was markedly impacted by sequence variation at C/EBP site II. The C/EBP site II 6G and ConB variants exhibited a high relative binding affinity with U-937 nuclear factors (Figure 1B). Conversely, the C/EBP site II 4C variant exhibited lower relative binding affinity with respect to binding U-937 nuclear factors (Figure 1B). C/EBP α and β antisera were utilized to determine which factors were recruited to the C/EBP site II ConB, 6G, and 4C variants. The C/EBP site II ConB and 6G variants both recruited C/EBP α and β proteins (Figure 1B), whereas the C/EBP site II 4C variant did not recruit detectable levels of C/EBP factors. However, an abundant DNA-protein complex was detected with a probe containing the 4C variant of C/EBP site II, although the identity of the protein components of this complex are unknown at the present time.

Low- and high-affinity C/EBP site I and II variants impacted basal LTR activation

We have previously demonstrated that the C/EBP binding site I 3T variant (C/EBP site I variant where position 3 contains a C to T nucleotide change from the consensus B sequence) recruited little, if any, C/EBP, whereas the C/EBP binding site I ConB variant recruited modestly higher levels of C/EBP factors (Hogan *et al*, 2003b; Ross *et al*, 2001a). In a similar manner, we report herein that the ConB and 6G variants of C/EBP binding site II recruited large quantities of C/EBP α and β, whereas the C/EBP binding site II 4C variant recruited little, if any, C/EBP α and β. As a first approach to examine the properties of these binding sites, recombinant LTR luciferase reporter constructs containing the C/EBP site II ConB, 6G, or 4C variants were constructed with either a C/EBP site I 3T or ConB configuration. The C/EBP site I 3T variant was of particular interest because it has been previously shown to appear only in brains of patients with HIVD and not in those from non-HIVD individuals (Hogan *et al*, 2003b). Transient transfection analyses were performed utilizing the U-937 monocytic cell line, and for comparative purposes, all values are shown relative to the construct containing the ConB sequence at both C/EBP sites I and II. Utilizing the C/EBP site II ConB variant, when C/EBP site I was changed from a modestly higher relative affinity sequence (ConB site I) to a low relative affinity sequence (3T site I), there was a 0.5-fold decrease in LTR activity. Utilizing the C/EBP site II 6G variant, when C/EBP site I was changed from the ConB to the 3T configuration, there was a 2.3-fold decrease in LTR activity. In striking contrast, utilizing the C/EBP site II 4C variant, when C/EBP site I was changed from a ConB to a 3T configuration, there was a 9.4-fold increase in LTR activity. In general, when C/EBP site I contained a low-affinity 3T variant, sequence variation at the C/EBP site II did not affect LTR basal activity. However, sequence variation at C/EBP site II had a dramatic affect on basal LTR activity in the presence of the ConB variant of C/EBP site I.

There is a difference in the regional distribution of LTRs containing the C/EBP site II ConB variant in the brains of HIV-1-infected patients with and without HIVD

We have previously demonstrated that 88.6% of all brain-derived LTRs (Ross *et al*, 2001a), 70% of brain-derived LTRs from patients with HIVD, and 94% of brain-derived LTRs from individuals without HIVD contain the ConB configuration of C/EBP site II (Hogan *et al*, 2003b). In addition, the C/EBP site II ConB variant recruited large quantities of C/EBP factors and is likely important in the regulation of the HIV-1 LTR during CNS disease. In conjunction with these studies, the regional distribution of LTRs containing the C/EBP site II ConB variant between brain tissue derived from patients with and without HIVD has also been examined. LTRs derived from seven patients with HIVD and three patients without HIVD in six brain regions were examined; mid-frontal gyrus (MFG), head of caudate (HC), deep white matter (DWM), parietal lobe (PAR), thalamus (THAL), and cerebellum (CRB). Utilizing the Fisher's exact test (Agresti, 1990), a statistically significant difference was found in the overall distribution of LTRs containing the C/EBP site II ConB variant in brain regions derived from patients with and without HIVD (P value = .00210) (Table 1). Two regions of the brain accounted for this overall difference in the distribution, mid-frontal gyrus (P value = .00578) and parietal lobe (P value = .02099) (Table 1). Overall, these results indicate that LTRs containing the C/EBP site-II

Table 1 Regional distribution of LTRs containing C/EBP site II ConB variant in brain tissues derived from HIV-1–infected individuals with and without dementia

Brain regions	*Demented*	*Nondemented*	*Total*	*Fisher's exact test* P *value*
CRB	12 17.91%	13 29.55%	25	.16938
DWM	7 10.45%	11 25.00%	18	.06375
HC	19 28.36%	12 27.27%	31	.99999
MFG	10 14.93%	0 0.00%	10	.00578*
PAR	8 11.94%	0 0.00%	8	.02099*
THAL	11 16.42%	8 18.18%	19	.80277
Total	67	44	111	
Overall P value = .00210*				

Note. Shown in the table are the number and percent of each LTR containing the given C/EBP site II variant in each of the six specific brain regions. Brain regions examined included mid-frontal gyrus (MFG), head of caudate (HC), deep white matter (DWM), parietal lobe (PAR), thalamus (THAL), and cerebellum (CRB).
* P values that are statistically significant.

ConB variant accumulated in the mid-frontal gyrus and parietal lobe of brains from individuals with HIVD.

There is a difference in the regional distribution of ConB, 6G, and 4C C/EBP site II variants derived from the brains of patients with HIVD

We have previously shown that the relatively low affinity C/EBP site II 4C variant and the relatively high affinity C/EBP site II 6G variant were only found in brains of patients with HIVD (Hogan *et al*, 2003b). In conjunction with these investigations, the regional distribution of LTRs containing the specific C/EBP site II variants in brain tissue derived from patients with HIVD was examined. LTRs obtained from six brain regions derived from seven patients with HIVD were also examined. As previously reported, there were no LTRs containing the C/EBP site II 4C or 6G variant found in brains of patients without HIVD (Hogan *et al*, 2003b). Therefore, regional distribution was not examined in brain tissues derived from non-demented individuals with these site II configurations. Utilizing Fisher's exact test (Agresti, 1990), a statistically significant difference was found in the overall distribution of LTRs containing the C/EBP site II ConB, 4C, and 6G variants in brain regions of patients with HIVD (P value $< .00001$) (Table 2). Three regions, cerebellum (P value $= .00001$), mid-frontal gyrus (P value $= .00005$), and head of caudate (P value $= .03117$), accounted for this overall difference in regional distribution of this group of HIV-1 LTRs containing C/EBP site II variants (Table 2). When the distribution within cerebellum of the LTRs containing the C/EBP site II 4C variant was compared to that of LTRs containing the C/EBP site II ConB variant, a P value of .00003 was obtained, suggesting there was a significantly higher prevalence of LTRs containing the C/EBP site II 4C variant in this region (data not shown). There was not a significant difference in the distribution of LTRs containing the C/EBP site II ConB variant when compared to LTRs containing the C/EBP site II 6G variant in the cerebellum (P value $=$ .34664, data not shown). Therefore, LTRs containing the C/EBP site II 4C variant were found to accumulate in a highly specific manner in the cerebellum. When the distribution of LTRs containing the C/EBP site II 6G variant compared to LTRs containing the C/EBP site II ConB variant was examined in the mid-frontal gyrus, a P value of .00007 was obtained, suggesting that there were significantly more LTRs containing the C/EBP site II 6G variant in this region (data not shown). There was not a significant difference in distribution of LTRs containing the C/EBP site II ConB variant when compared to LTRs containing the C/EBP site II 4C variant in the mid-frontal gyrus (P value $=$ .58338, data not shown). Therefore, LTRs containing the C/EBP site II 6G variant were found to accumulate in the mid-frontal gyrus with a lower level of accumulation observed in the deep white matter. In addition, the LTRs containing the C/EBP site II ConB variant were most significantly found in the head of caudate (Table 2). In conclusion, viruses with distinct LTR populations containing C/EBP site II variants were found in different neuroanatomical regions of the brain.

Table 2 Regional distribution of LTRs containing C/EBP site II 6G, 4C, or ConB variants in brain tissues derived from HIV-1–infected individuals with dementia

Brain regions	*C/EBP II 4C variant*	*C/EBP II 6G variant*	*C/EBP II ConB variant*	*Total*	*Fisher's exact test* P *value*
CRB	7 100.00%	0 0.00%	12 17.91%	19	.00001*
DWM	0 0.00%	2 20.00%	7 10.45%	9	.41730
HC	0 0.00%	0 0.00%	19 28.36%	19	.03117*
MFG	0 0.00%	8 80.00%	10 14.93%	18	.00005*
PAR	0 0.00%	0 0.00%	8 11.94%	8	.64028
THAL	0 0.00%	0 0.00%	11 16.42%	11	.32394
Total	7	10	67	84	
	Overall P value $< .00001$*				

Note. Shown in the table are the number and percent of each LTR containing the given C/EBP site II variant in each of the six specific brain regions. Brain regions examined included mid-frontal gyrus (MFG), head of caudate (HC), deep white matter (DWM), parietal lobe (PAR), thalamus (THAL), and cerebellum (CRB).
* P values that are statistically significant.

Discussion

HIV-1 sequence variation within the LTR as well as other viral genes, such as *env*, affects viral replication and viral tropism, which ultimately may impact disease progression. Studies have demonstrated that HIV-1 LTRs evolve in a compartmentalized manner (Ait-Khaled and Emery, 1994; Ait-Khaled *et al*, 1995). Thus, HIV-1 LTR sequence variation may indeed play a role in adaptation of the virus to the CNS as well as to other tissues. In the studies reported herein, the regional distribution of LTRs containing sequence variants at C/EBP site II in different brain regions was examined. Based on previous observations, it has been demonstrated that the cerebellum is not usually a site of productive viral replication. The studies reported herein demonstrated that LTRs containing the low-affinity C/EBP site II 4C variant accumulated in the cerebellum. Consistent with this observation, additional studies have demonstrated that LTRs containing the C/EBP site II 4C configuration exhibit a low basal activity in transient expression assays performed in U-937 monocytic cells, particularly in the presence of a ConB variant of C/EBP site I. We hypothesize that LTRs containing the 4C configuration at C/EBP site II and the ConB configuration at C/EBP site I may lead to an integrated

provirus that would exhibit a low basal transcriptional activity that may facilitate the establishment and maintenance of a silent genome. These studies are clearly consistent with the lack of HIV-1 gene expression and viral activity in the cerebellum of HIVD brains.

The mid-frontal gyrus, which is involved in cognitive processes, typically is productively infected with HIV-1 (Glass *et al*, 1995). In addition, it has been shown to be a site of inflammation with an abundance of macrophages readily detected. Our studies demonstrated that LTRs containing the high-affinity C/EBP site II 6G variant were found to accumulate in the mid-frontal gyrus, a site of productive viral replication with the formation of multinucleated giant cells, and these LTRs also exhibited high basal activities. Based on inherent selective pressures in the mid-frontal gyrus, LTRs containing C/EBP site II 6G variants accumulated in this region. In summary, we have demonstrated an association between LTRs with high basal activities, particularly those with the 6G configuration at C/EBP site II and the ConB C/EBP site I configuration and their presence in a HIVD brain region in which productive viral replication has been readily detected.

The accumulation of LTRs containing the C/EBP site II 6G variant in the mid-frontal gyrus of patients with HIVD may be important in the development of the cognitive defect associated with HIVD. The mid-frontal gyrus is located in the frontal region, which is involved in motor function, problem solving, spontaneity, memory, language, initiation, impulse control, and social and sexual behavior. In addition, the mid-frontal gyrus is typically productively infected with HIV-1 and is a site of inflammation with an abundance of macrophages readily detected. In this region, LTRs containing the C/EBP site II 6G variant with high basal activities would be activated by C/EBP-related factors. Viruses containing LTRs with either the C/EBP site II 6G variant in the mid-frontal gyrus would potentially lead to productive viral replication, inflammation, the formation of multinucleated giant cells, and impairment of cognitive functions leading to HIVD.

In general, the results reported herein concerning the regional distribution of particular LTR genotypes within the brains of demented and nondemented individuals are consistent with previous reports (discussed in the Introduction) concerning the regional distribution of the *env* sequence variation. Several studies have demonstrated genetically unique *env* variants present in different regions of the brain, including the frontal and parietal lobes (Chang *et al*, 1998; Shapshak *et al*, 1999).

There are three models that could account for the occurrence of brain region-specific distribution of HIV-1 LTRs or other viral sequences in infected individuals. In the first model, seeding of the brain by free virus or more likely infected macrophages would involve viral entry into the brain primarily during the initial infection. Subsequent to CNS entry, HIV-1 variants would only evolve due to region-specific selective pressures unique to different microenvironments of the brain. This model seems to be the most likely method that intra-CNS evolution (Major *et al*, 2000); however, the role of intra-CNS evolution plays in HIVD is not clear.

In a second model, virus may enter the brain at multiple time points after the initial infection and may subsequently spread to other brain regions. Viral entry into the brain could take place repeatedly utilizing the same entry pathway; however, it would be more likely that the virus would utilize multiple entry pathways during the course of progressive disease. In this case, brain region–specific distribution would be due not only to inherent differences in brain microenvironment, but also to periodic changes to brain microenvironments after initial infection such as alterations in chemokine and cytokine pathways. In addition, changes in the viral genomic structure during the course of infection could impact the nature of virus initially entering the CNS at a given time point, which could impact regional distribution. Previous data from our laboratory (Hogan *et al*, 2003b) demonstrated that peripheral blood-derived LTRs containing either the C/EBP site II 6G or the 4C variant were only found at early stage HIV-1 infection. Here, we demonstrated that these variants were only found in the brain tissues of patients with HIVD. Because LTRs containing either the C/EBP site II 6G or the 4C variant were only found at early stage HIV-1 infection in the peripheral blood, viruses containing these LTRs had to transverse the blood-brain barrier early in disease, because they were not found in the blood later in disease progression.

In the final model, virus infection of the CNS could involve multiple brain entry pathways leading to the delivery of viral genomes containing unique LTR sequences to specific regions of the brain. This would be more likely to occur during the later stages of infection, when there is an increased infiltration of macrophages across the blood-brain barrier. In support of this model, it has been suggested that HIVD is primarily due to an increase in the trafficking of bone marrow–derived blood monocytes, both HIV infected and uninfected, into the brain during the later stages of HIV disease (Gartner, 2000). In one study of a patient with HIVD, close genetic relatedness among HIV-1 gp160 sequences recovered from circulating monocytes, bone marrow, and a subcortical region of brain was observed, whereas sequences from other tissues, including lung and lymph node, clustered independently (Liu *et al*, 2000). If the development of HIVD is a consequence of HIV species entering the brain late in the course of infection, then evolutionary pressures with pathological potential might exert their effects outside of the brain, that is, prior to the trafficking of the infected cells into the brain. This could include pressures that lead to selection of particular genetic motifs within the LTR, including,

motifs within regions associated with control of virus expression. Thus, evolution of the HIV-1 LTR in this scenario would be driven by selective pressures within cells of the peripheral immune system and perhaps bone marrow and not within brain tissue of HIV-1–infected patients these sequences would be distributed in the brain late in infection. Based on these observations, future studies will examine HIV-1 LTR sequence variation at C/EBP sites I and II at late stage disease in peripheral blood, bone marrow, and brain of patients with and without HIVD. Viruses containing LTRs with C/EBP variants, other than 4C and 6G site II, could transverse the blood-brain barrier later in disease (because in the blood at late stage disease there are no 4C or 6G site II variants found [Hogan *et al*, 2003b]) and evolve in the mid-frontal gyrus to contain a C/EBP site II 6G variant and in the cerebellum to contain a C/EBP site II 4C variant. Further studies are currently underway in order to determine the origin of the specific brain region variants.

Materials and methods

Cell culture and nuclear extract preparation
The U-937 (ATCC no. CRL-1593.2) human monocytic cell line was grown in RPMI 1640 medium. The medium was supplemented with 10% heat-inactivated fetal bovine serum, antibiotics (penicillin, streptomycin, and kanamycin, each at a concentration of 0.04 mg/mL), L-glutamine (0.3 mg/ml), and sodium bicarbonate (0.05%). The cells were maintained at 37°C in 5% CO_2 at 90% relative humidity. Nuclear extracts were prepared as described (Dignam *et al*, 1983).

Oligonucleotide synthesis, radiolabeling, and EMS analyses
Complementary single-stranded oligonucleotides corresponding to C/EBP site II sequences (ConB, 4C, and 6G) were synthesized (Macromolecular Core Facility, Penn State College of Medicine, Hershey, PA) and annealed by brief heating at 100°C followed by slow cooling to room temperature. Blunt-ended, double-stranded oligonucleotides were end-labeled using [$\gamma-^{32}$P]-ATP and T4 polynucleotide kinase as described (Promega).

EMS reactions were performed using 75,000 CPM of radiolabeled, double-stranded oligonucleotide, U-937 nuclear protein extract (20 μg), and poly[d(I-C)] (1 μg), in a total reaction volume of 15 μl. DNA-protein complexes were allowed to form at 30°C for 20 min. In supershift EMS reactions, antibody (final concentration of 70 ng/μl) was added to the reactions and the reactions were allowed to proceed for an additional 20 min at 30°C. Subsequent to completion of all reactions, electrophoresis (30 mA and 200 V) was performed in a 5% high-ionic-strength native polyacrylamide gel. All antibodies used were obtained through Santa Cruz Biotechnology.

Plasmids, site-directed mutagenesis, and transient expression analyses
The parental LAI luciferase construct was used as a template for site-directed mutagenesis as described previously (Hogan *et al*, 2003b). The QuickChange mutagenesis kit (Stratagene, La Jolla, CA) was used to construct luciferase plasmids with changes in C/EBP sites I and II. All plasmids used in these studies were sequenced to verify the C/EBP binding site sequence configurations (Macromolecular Core Facility, Penn State College of Medicine). Transient expression analyses were performed as described previously (Hogan *et al*, 2003b).

PCR amplification of brain tissue
Amplification of brain regions was performed as described (Hogan *et al*, 2003b). Brain regions examined included mid-frontal gyrus (MFG), head of caudate (HC), deep white matter (DWM), parietal lobe (PAR), thalamus (THAL), and cerebellum (CRB).

Statistical analyses
Fisher's exact test was performed as described in Agresti (1990) to determine if there was a difference in regional distribution of C/EBP site II sequences. *P* values less than or equal to .05 are considered statistically significant values.

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