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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

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The appearance and progression of human immunodeficiency virus type 1 (HIV-1)–associated pathogenesis in the immune and central nervous systems is dependent on the ability of the virus to replicate in these compartments, which is, in turn, controlled by numerous factors, including viral binding and entry, receptor and coreceptor usage, and regulation of viral expression by the long terminal repeat (LTR). The LTR promotes viral expression in conjunction with viral and cellular regulatory proteins, including members of the CCAAT/enhancer binding protein (C/EBP) family, which modulate LTR activity through at least two *cis*-acting binding sites. Previous studies have shown that these sites are necessary for HIV-1 replication in cells of the monocyte/macrophage lineage, but dispensable in T lymphocytes. To establish potential links between this important family of transcription factors and HIV-1–associated pathogenesis, C/EBP site I and II sequence variation in peripheral blood mononuclear cell (PBMC)-derived LTRs from HIV-1–infected patients with varying degrees of disease severity was examined. A high prevalence of C/EBP site variants 3T (site I) and consensus B (site II) within PBMC-derived HIV-1 LTRs was shown to correlate with late stage disease in HIV-1–infected patients. These results suggest that the increased prevalence in the PBMCs of HIV-1 LTRs containing the 3T C/EBP site I variant and the consensus B site II variant may serve as a molecular marker for disease progression within the immune system. The relative low or high binding affinity of C/EBP γ to sites I and II in electrophoretic mobility shift (EMS) analyses correlated with low or high LTR activity, respectively, in transient expression analyses during both early and late disease stages. The 3T C/EBP site I was the only variant examined that was not found in LTRs derived from PBMCs of patients at early stages of HIV-1 disease, but was found at increasing frequencies in patients with late stage disease. Furthermore, the 3T C/EBP site I was not found in brain-derived LTRs of patients without HIV-1–associated dementia (HIVD), but was found in increasing numbers in brain-derived LTRs from patients diagnosed with HIVD.

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The C/EBP site I 3T variant appears to be exclusive to patients progressing to increasingly severe HIV-1–associated immunologic and neurologic disease.
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Introduction

Human immunodeficiency virus type 1 (HIV-1)–associated immunologic and neurologic disease is dependent on the ability of the virus to infect resident immune and central nervous system (CNS) cell populations. *In vitro* and *in vivo* investigations have shown that HIV-1 infection of $CD4^+$ T lymphocytes initiates a highly productive infection in stimulated T-cell populations (Pantaleo *et al*, 1993). In contrast, HIV-1–infected monocytic cell populations produce only limited quantities of virus within the context of a more chronic infection (Gendelman *et al*, 1989; Levy, 1993). The chronic nature of HIV-1 replication in cells of monocyte/macrophage lineage is a likely contributor to the central importance of these cells in evasion of HIV-1 detection by the immune system and the maintenance of viral reservoirs (Martin and Bandres, 1999). Other studies have shown that HIV-1 infection of the monocytic lineage of cells plays key roles in CNS invasion (Fischer-Smith *et al*, 2001; Gartner and Liu, 2002; Gartner *et al*, 1986; Liu *et al*, 2000; Williams *et al*, 2001) and in the genesis of HIV-1–associated dementia (HIVD; for review, see Krebs *et al*, 2000).

HIV-1 viral gene expression in monocytes and macrophages (as well as T cells and other cell types susceptible to infection) is critically dependent on regulation of the long terminal repeat (LTR), the promoter element that drives expression from proviral DNA and guides the synthesis of potentially toxic viral proteins and infectious virus. Factors that influence the level of LTR-directed transcription include sequence variation within *cis*-acting regulatory elements, alteration of the levels and specific activities of cellular transcription factors during the course of cellular differentiation and activation, and the complex network of signaling pathways that converge on the LTR (for review of LTR, see Krebs *et al*, 2001). The LTR, in turn, relies heavily on participation of viral factors, such as Tat and Vpr, as well as cellular transcription factors, including nuclear factor μB (NF-μB), Sp, activating transcription factor/cyclic AMP response element binding protein (ATF/CREB) (Gowda *et al*, 1989; Krebs *et al*, 1997, 1998; McDougal *et al*, 1985), and members of the CCAAT/enhancer binding protein (C/EBP) family (Henderson and Calame, 1997; Henderson *et al*, 1995, 1996; Ross *et al*, 2001a, 2001b).

C/EBP factors are intimately involved in the regulation of monocyte- and/or myeloid-specific gene expression (Bretz *et al*, 1994; Matsusaka *et al*, 1993; Pope *et al*, 1994; Stein and Baldwin, 1993; Tanaka *et al*, 1995). The C/EBP family includes at least eight different proteins, many of which are important activators, such as C/EBP μ (Birkenmeier *et al*, 1989), C/EBP μ (also called liver activator protein or LAP), and NF–interleukin-6 (NF–IL-6) (Akira *et al*, 1990; Chang *et al*, 1990; Descombes *et al*, 1990), and repressors of transcription, such as liver inhibitor protein (LIP), a truncated form of C/EBP μ. C/EBP proteins are members of the b-ZIP family of transcription factors, which share a highly homologous carboxyl-terminus that contains the basic and leucine zipper domains. C/EBP family members homo- and heterodimerize through their leucine zippers, and bind to their cognate DNA sequence through their corresponding basic regions. C/EBP factors also interact with other factors, including Sp (Lee *et al*, 1997), NF-μB (Stein and Baldwin, 1993), and ATF/CREB (Ross *et al*, 2001b; Vallejo *et al*, 1993), to synergistically activate transcription of a number of eukaryotic and viral promoters, including the HIV-1 LTR. C/EBP μ binds to at least two sites within the HIV-1 LTR and has been shown to activate viral transcription in transient transfection analyses (Tesmer *et al*, 1993) (see Figure 1). 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).

C/EBP regulation of HIV-1 viral gene expression in cells of the monocytic and macrophage lineage is modulated by sequence variation that occurs

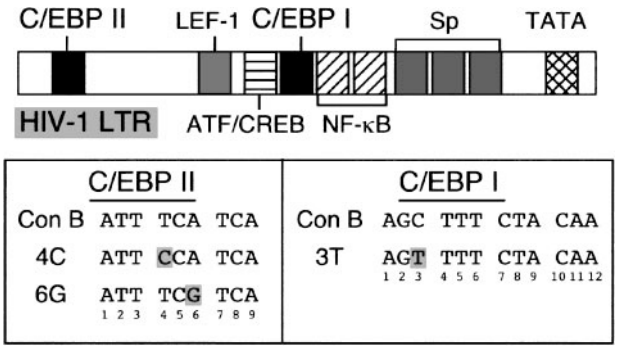

Figure 1 *Cis*-acting elements of the HIV-1 LTR and C/EBP site variants are shown. The upper panel is a pictorial representation of the HIV-1 LTR with *cis*-acting elements, including C/EBP, LEF-1, ATF/CREB, NF-μB, and Sp sites, and TATA box. Sequence variants 3T and Con B C/EBP site I and Con B, 6G, and 4C C/EBP site II are shown in the lower panel. All changes from consensus B are in a shaded box.

naturally within the C/EBP sites and throughout the proviral genome. Varying levels of viral replication in peripheral blood mononuclear cells (PBMCs) and lymphoid tissues results in the appearance of a spectrum of related but distinct viral genotypes (quasispecies) as a result of nucleotide misincorporations into the proviral DNA genome (Goodenow *et al*, 1989). These HIV-1 sequence variants typically possess divergent cellular tropisms, virulence, and degrees of infectivity. Through this lengthy process, HIV-1 can adapt to different host cells and spread into niches that a single viral strain would be unable to occupy. The prevalence of specific viral sequence variants is a consequence of positive and negative selective pressures applied by the immune system during the course of disease. The accumulation of specific LTR sequences over time may also result from accumulation of poorly replicating viruses or latent proviruses in long-lived cell subsets in the circulation or in viral reservoirs, such as resting memory $CD4^+$ T cells (Blankson *et al*, 2002).

Recent studies demonstrated that specific HIV-1 LTR C/EBP configurations that arise as a consequence of quasispecies evolution were preferentially encountered in the brain and exhibited enhanced LTR-directed transcriptional activity (Ross *et al*, 2001a). Transient expression analyses indicated that inclusion of a C/EBP site I with high affinity for C/EBP factors resulted in increased basal and IL-6–induced LTR activity. These studies demonstrated that brain-derived LTRs contain two relatively high affinity C/EBP binding sites, which suggests that these sites may play an important role in LTR-directed transcription during the course of CNS disease (Ross *et al*, 2001a).

However, to date, direct examination of LTR sequences with respect to disease progression has failed to reveal any substantive associations. A number of studies have compared LTR sequences from HIV-1–positive long-term nonprogressor and rapid progressor cohorts (Berkhout and Jeang, 1992; Quinones-Mateu *et al*, 1998; Rousseau *et al*, 1997; Zhang *et al*, 1997). In each case, no simple correlation between LTR sequence variants and the rapidity of disease onset was identified. Moreover, in two of the three studies, transcriptional activities of variant LTRs were examined by transient expression analyses in both cell lines and PBMCs (Quinones-Mateu *et al*, 1998; Zhang *et al*, 1997). These studies indicated no significant relationship between promoter strength and disease progression. However, there were two individual nonprogressors who exhibited potentially defective LTRs. LTR sequences from one patient in the Zhang cohort (one of eight) contained G to A hypermutations throughout the promoter structure, suggesting a defective 5′ LTR (Zhang *et al*, 1997). Similarly, a long-term nonprogressor from the Rousseau study (one of four) had an array of insertions and deletions across the LTR, again suggesting a potentially defective LTR structure (Rousseau *et al*, 1997). These observations suggest that defective LTR structures may predict long-term nonprogression.

Other studies suggest that more active LTRs may be linked to greater infectivity and viral propagation throughout the population at risk for HIV-1 infection. HIV-1 clade C LTRs, which have three NF-μB sites, have increased LTR activation as compared to those with only one or two NF-μB sites. Viral replication studies demonstrated that subtype C viruses produced increased p24 levels and thus higher replication rates than viruses of other clades (Naghavi *et al*, 1999). The presence of three NF-μB binding sites may be linked to the more rapid spread of subtype C viruses compared to any other subtypes (Rodenburg *et al*, 2001).

The important roles that monocytic cells play in HIV-1 infection of the immune system and CNS strongly suggest that these cells are determinants in the progression and outcome of HIV-1–associated disease. Their roles in influencing the course of disease are also likely subject to the effects of HIV-1 sequence diversity, the generation of which has been linked to the rapidity of pathogenesis (Berkhout and Jeang, 1992; Zhang *et al*, 1997). Because of the integral involvement of C/EBP factors in the regulation of HIV-1 replication in cells of monocyte/macrophage lineage, we hypothesized that sequence variation within C/EBP sites I and II of the HIV-1 LTR might influence the progression of HIV-1 disease in the PBMC population, lymphoid tissues, and brain by modulating LTR activity, viral gene expression, and HIV-1 replication. The studies presented herein describe correlations between specific C/EBP site sequence variants in PBMCs- and brain-derived LTRs and the severity of HIV-1–associated immunologic dysfunction and neuropathogenesis. These results also suggest that specific alterations in LTR sequence may be used as markers for disease progression in HIV-1–infected patients.

Results

C/EBP sites I and II from patients with varying disease severity were analyzed with respect to sequence variation

Sequence analyses of C/EBP sites I and II were performed using HIV-1 LTR sequences derived from PBMCs documented in three published studies (Estable *et al*, 1996; Kirchhoff *et al*, 1997; Michael *et al*, 1994). These analyses were performed with regard to the donor's disease state and severity documented in each of the three studies. Because each study described patient disease severity using a different classification system, LTRs were assigned to one of three groups prior to analysis. Sequences that were originally cloned from patients with HIV-1 infections characterized as asymptomatic, nonprogressing, or stage I (World Health Organization

Table 1 Clinical disease states, number of patients, and PBMC-derived LTR sequences examined at C/EBP site I and /or C/EBP site II

Disease severity group (DSG)	*Clinical stage*	*Number of patients*	*Number of LTRs*	*C/EBP site(s) examined*	*Reference*
1	Asymptomatic	4	78	I and II	Michael *et al*, 1994
	Stage I	4	12	I and II	Estable *et al*, 1996
	Nonprogressing	5	44	I	Kirchhoff *et al*, 1997
2	Stage II	12	65	I and II	Estable *et al*, 1996
	Stage III	10	40	I and II	Estable *et al*, 1996
	Slow-progressing	4	17	I	Kirchhoff *et al*, 1997
3	Stage IV	10	73	I and II	Estable *et al*, 1996
	Progressing	4	19	I	Kirchhoff *et al*, 1997

Note. PBMC-drived LTR sequences were obtained from three published studies (Estable *et al*, 1996; Kirchhoff *et al*, 1997; Michael *et al*, 1994). Patients were organized into three disease severity groups (DSGs). DSG 1 included LTRs derived from patients who were characterized as asymptomatic, nonprogressing, or WHO classification stage I (Kassa *et al*, 1999; WHO, 1993). DSG 2 included LTRs derived from patients who were characterized as slow-progressing, stage II, or stage III. DSG 3 included LTRs derived from patients who were characterized as fast-progressing or stage IV. In short, patients in DSG 1 had the least severe disease, whereas patients in DSG 3 were diagnosed as having the highest disease severity. Sequence variation at C/EBP site I and C/EBP site II was examined in LTRs from all three DSGs.

[WHO], 1993; Kassa *et al*, 1999) were grouped under disease severity group 1 (DSG 1). DSG 1 was made up of a total of 13 patients (4, 4, and 5) and a total of 134 LTRs (78, 12, and 44, respectively) (Table 1). Disease severity group 2 (DSG 2) consisted of sequences from patients with HIV-1 infections characterized as slow-progressing, stage II or III (WHO, 1993; Kassa *et al*, 1999). DSG 2 consisted of a total of 26 patients (12, 10, and 4) and 122 LTRs (65, 40, and 17, respectively) (Table 1). Disease severity group 3 (DSG 3) was comprised of sequences that were originally cloned from patients with HIV-1 infections characterized as progressing or stage IV (WHO, 1993; Kassa *et al*, 1999). DSG 3 consisted of a total of 14 patients (10 and 4) and 92 LTRs (73 and 19, respectively) (Table 1). A total of 53 patients and 348 LTR sequences were used in these studies. Because C/EBP site II sequence information was not available in the Kirchhoff study, DSGs 1, 2, and 3 with respect to site II contained only 89, 105 and 73 LTRs, respectively.

Prevalence of LTRs containing the 3T C/EBP site I increased dramatically with disease progression, whereas the frequency of consensus B C/EBP site I decreased

HIV-1 PBMC-derived LTRs were analyzed for C/EBP site I sequence variation. The prevalence of LTRs containing the consensus B C/EBP site I (AGC TTT CTA CAA) (Figure 1) decreased significantly in the PBMCs of patients with late stage HIV-1 disease (chi-square analysis resulted in a P value of $<.0001$) (Figure 2), whereas the frequency of LTRs containing the 3T C/EBP site I (AGT TTT CTA CAA; changes from consensus B C/EBP site I are underlined) significantly increased as disease progressed (chi-square analysis resulted in a P value of $<.0001$) (Figure 2). Specifically, the frequency of consensus B site I decreased progressively from DSG 1 to DSG 3 (DSG 1, 68.7%; DSG 2, 52.5%; DSG 3, 32.6%) (Figure 2). On the other hand, the prevalence of the 3T site I variant, which was not present at all in DSG 1, increased to 8.2% in DSG 2 and 47.8% in DSG 3 (Figure 2). Because the LTRs examined in each of the DSGs are LTRs from a compilation of a number of different patients with that same disease severity, we have now begun longitudinal studies to determine the course of C/EBP alterations over time in individual patients.

LTRs containing the consensus B C/EBP site II increased dramatically with disease progression as the frequency of 6G and 4C C/EBP site II decreased

In parallel with studies focused on C/EBP site I sequence variation, sequence variation at site II was also explored within the HIV-1 LTRs derived from PBMCs. The prevalence of LTRs containing consensus B C/EBP site II (ATT TCA TCA) was significantly increased in the PBMCs of patients with late stage HIV-1 disease (chi-square analysis resulted in a P value of $<.0001$), whereas LTRs containing 4C (ATT CCA TCA, changes from consensus B C/EBP site II are underlined) and 6G (ATT TCG TCA) C/EBP site II decreased significantly (P values for both the 4C and 6G configurations were $<.0001$) (Figure 3). Specifically, the frequency of consensus B site II progressively increased from DSGs 1 to 3 (23.6%, 57.1%, 93.2%, respectively) (Figure 3). On the other hand, the prevalence of the 4C site II variant, which was present in 28.1% of LTRs in DSG 1, decreased to 7.6% in DSG 2 and was absent in DSG 3 (Figure 3). Similarly, the prevalence of 6G site II variant was dramatically decreased from DSGs 1 to 3 (34.8%, 14.2%, 1.4%, respectively).

NF-μB site II was highly conserved in LTRs derived from patients with disease of increasing severity

As a control for these studies, sequence variation within the NF-μB site II (promoter distal) was examined. NF-μB site II was selected because it is positioned adjacent to C/EBP site I and is also a site important to HIV-1 LTR function in immune cell populations. NF-μB site II was found to be highly conserved in DSGs 1, 2, and 3 (92.5%, 97.5%, and

	C/EBP site I sequence	Number of LTRs	Percent of total
DSG 1	CONB	92	68.7
	3A	26	19.4
	2A	5	3.7
	1G3C6C7A9A	5	3.7
	3T	0	0.0
	OTHER	6	4.5
	TOTAL	134	100
DSG 2	CONB	64	52.5
	6G	14	11.5
	3T	10	8.2
	10T	9	7.3
	9G	6	4.9
	1C3G4C	6	4.9
	1C	5	4.1
	OTHER	8	6.6
	TOTAL	122	100
DSG 3	CONB	30	32.6
	3T	44	47.8
	5A	6	6.5
	1C5C	3	3.3
	3A	2	2.2
	1C	2	2.2
	OTHER	5	5.4
	TOTAL	92	100

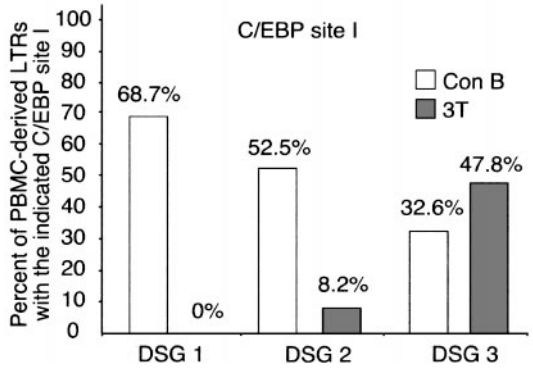

Figure 2 The prevalence of consensus B C/EBP site I decreased in LTRs from PBMC derived from patients in late stage HIV-1 disease, whereas 3T C/EBP site I increased with disease progression. Sequence variation at C/EBP site I was examined in LTRs derived from PBMCs of patients with increasing degrees of HIV-1 disease progression. Sequence variation at C/EBP site I within 134, 122, and 92 LTRs was examined in DSG 1, 2, and 3, respectively. The table includes the DSG, site sequence, the number of LTRs in which that sequence was found, and the prevalence (percent) of that site within the population of LTRs in that DSG. The graph depicts the prevalence of Con B and 3T C/EBP site I variants in LTRs derived from patients in DSG 1, 2, and 3. The prevalence of consensus B C/EBP site I decreased in LTRs derived from PBMC and the prevalence of the 3T C/EBP site I increased with increasing disease severity.

	C/EBP site II sequence	Number of LTRs	Percent of total
DSG 1	CONB	21	23.6
	6G	31	34.9
	4C	25	28.1
	5A	2	2.2
	1C	2	2.2
	OTHER	8	9.0
	TOTAL	89	100
DSG 2	CONB	60	57.1
	6G	15	14.3
	4C	8	7.6
	7G	8	7.6
	9C	6	5.7
	1T4C	6	5.7
	1C4C	2	2.0
	TOTAL	105	100
DSG 3	CONB	68	93.1
	7G	3	4.1
	6G	1	1.4
	4A	1	1.4
	4C	0	0.0
	TOTAL	73	100

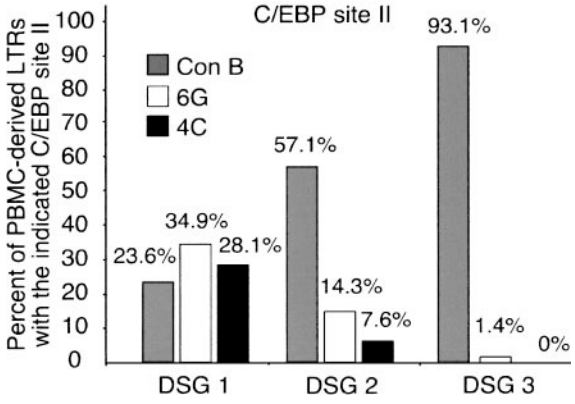

Figure 3 The prevalence of consensus B C/EBP site II increased in LTRs derived from PBMC of patients in late stage HIV-1 disease, whereas 6G and 4C C/EBP site II decreased with disease progression. Sequence variation at C/EBP site II was examined in LTRs derived from PBMC of patients with increasing HIV-1–associated disease severity. A total of 89, 105, and 73 LTRs were examined for sequence variation at C/EBP site II in patients in DSG 1, 2, and 3, respectively. The table includes the DSG, site sequence, the number of LTRs in which that sequence was found, and the prevalence (percent) of that site within the population of LTRs in that DSG. The graph depicts the prevalence of Con B, 6G, and 4C C/EBP site II variants in LTRs derived from patients in DSGs 1, 2, and 3. The prevalence of consensus B C/EBP site II increased in LTRs derived from PBMC and the prevalence of the 6G and 4C C/EBP site II decreased with increasing disease severity.

99%, respectively) (Figure 4). A Fisher's exact test was used to determine if there was any significant change in the frequency of consensus B NF-μB site II over the course of disease severity. The *P* value for this analysis was .6363, indicating no association between disease severity and NF-μB site II variation. These results indicate that NF-μB site II, a *cis*-acting site that also supports HIV-1 viral gene expression in monocytic cells, does not vary with increasing disease severity.

LTRs from patients with and without HIVD possess different C/EBP site I and II sequence variants

To determine if any correlations could be established between disease severity and sequence variation of brain-derived LTRs, sequence variation at C/EBP sites I and II was examined in LTRs derived from postmortem brain tissues of patients with and without HIVD. Patients that were characterized in DSG 3 were the most likely to exhibit neurologic signs and symptoms associated with HIVD. According to the WHO staging system of HIV infection, patients with stage IV disease (represented in DSG 3) may include those diagnosed with HIV-1 encephalopathy and dementia (Kassa *et al*, 1999; WHO, 1993). However, in the three studies utilized in this analysis (Estable *et al*, 1996; Kirchhoff *et al*, 1997; Michael *et al*, 1994), the prevalence of HIV-1–associated neurologic disease was not reported. LTRs with consensus B or 3T C/EBP site I or consensus B, 4C, or 6G C/EBP site II configurations were selected from LTRs isolated from brain tissues from demented and nondemented patients. In Figure 5 (**A** and **B**), each bar indicates the frequency (percent) of the sequence variant within each LTR population. Similar to what has been observed in LTRs derived from PBMCs at increasing disease severity, the 3T site I variant was absent within patients without dementia. However, 25% of LTRs derived from patients with HIVD had the 3T C/EBP site I configuration (Figure 5**A**) (chi-square analysis resulted in a *P* value of <.000001). The prevalence of the consensus B site I variant was 23% in brain-derived LTRs from patients without HIVD, and increased to 57% in LTRs derived from patients with HIVD (Figure 5**A**) (chi-square analysis resulted in a *P* value of .00008). Relative to C/EBP site II, the prevalence of the consensus B variant was 94% in brain-derived LTRs from patients without HIVD, and decreased to 70% in LTRs derived from patients with HIVD (Figure 5B) (chi-square analysis resulted in a *P* value of .00001). Similarly, neither the 6G nor 4C C/EBP site II variants were present in LTRs from nondemented patients, but increased in frequency to 10% and 7%, respectively, in demented patients (Figure 5**B**) (chi-square analysis resulted in a *P* value of <.000001).

	NF-κB site II sequence	Number of LTRs	Percent of total
DSG 1	CONB	124	92.5
	1A	8	6.0
	OTHER	2	1.5
	TOTAL	134	100
DSG 2	CONB	119	97.5
	1A	0	0.0
	OTHER	3	2.5
	TOTAL	122	100
DSG 3	CONB	91	99.0
	1A	0	0.0
	OTHER	1	1.0
	TOTAL	92	100

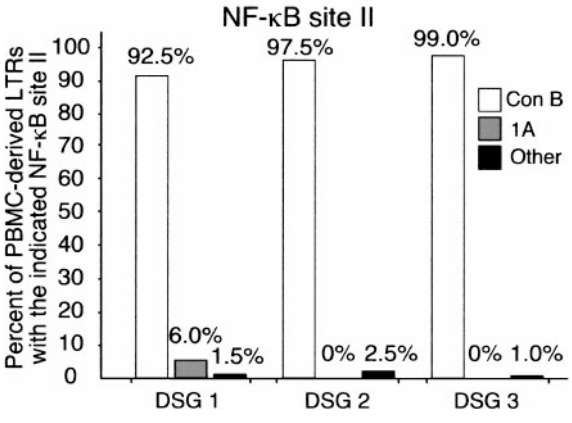

Figure 4 NF-μB site II is highly conserved in LTRs derived from PBMCs of patients across different stages of HIV-1–associated disease. Sequence variation at NF-μB site II was examined and the number and percent of LTRs with each configuration is shown in the table. Sequence variation at NF-μB site II within 134, 122, and 92 LTRs was examined in DSG 1, 2, and 3, respectively. The table includes the DSG, site sequence, the number of LTRs in which that sequence was found, and the prevalence (percent) of that site within the population of LTRs in that DSG. The graph depicts the prevalence of all NF-μB site II variants in LTRs derived from patients in DSGs 1, 2, and 3. The prevalence of the consensus B NF-μB site II remained relatively constant throughout the course of disease.

C/EBP sites I and II have different relative affinities for purified C/EBP μ protein

To examine the impact of C/EBP site sequence variation on C/EBP μ protein recruitment and to quantitate the differences in relative C/EBP μ binding, competition electrophoretic mobility shift (EMS) analyses were performed utilizing C/EBP site I and II variants as competitors (Figures 6**A** and **B**, respectively). An oligonucleotide probe spanning the consensus B C/EBP site II was radiolabeled and reacted with purified C/EBP μ protein to make comparisons between site I and II affinities. The 6G C/EBP site I variant, which is present in the LAI LTR, required an 80-fold molar excess competitor to reduce DNA-protein complex formation by 50% and exhibited the highest affinity for C/EBP μ at site I (Figure 6**A**). Consensus B and 3T site I variants required over 100-fold molar excess competitor to reduce DNA-protein

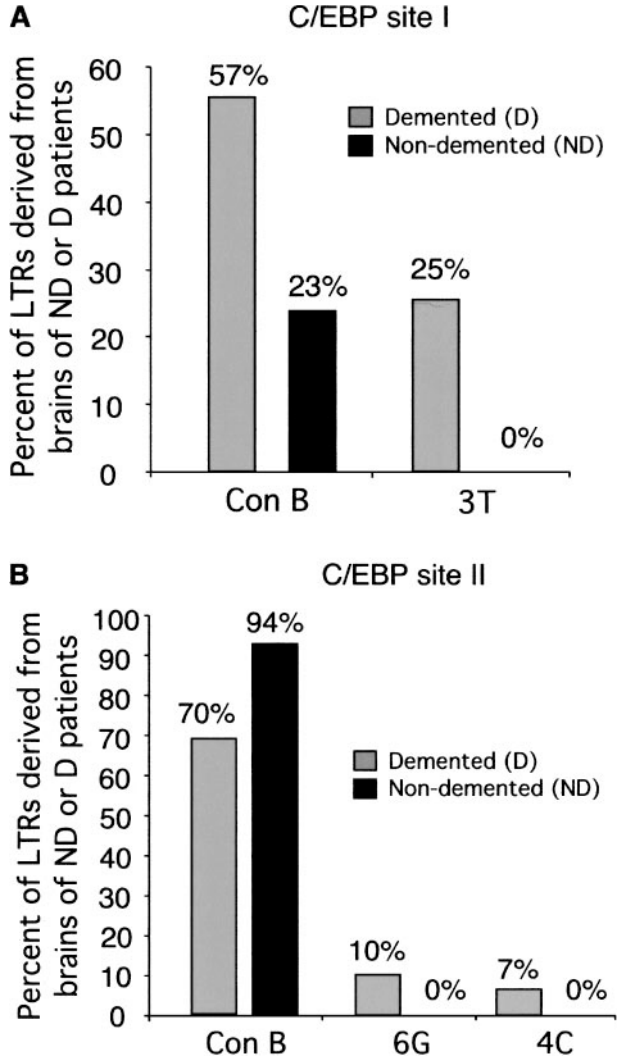

Figure 5 C/EBP site I and II sequence variation in LTRs derived from brain tissue of patients with and without HIVD. (**A**) Sequence variation at C/EBP site I was examined in LTRs derived from patients with and without HIVD. Consensus B site I was found in 57% of LTRs derived from patients with HIVD and 23% of nondemented LTRs. The 3T site I variant was found in 25% of LTRs derived from demented patients and was not found in LTRs derived from patients without HIVD. (**B**) Sequence variation at C/EBP site II was examined from LTRs derived from patients with and without HIVD. Consensus B site II was found in 70% of LTRs from patients with HIVD and 94% of LTRs from patients without HIVD. 6G site II was found in 10% of LTRs from patients with HIVD and no LTRs from patients without HIVD. 4C site II was found in 7% of LTRs from patients with HIVD and no LTRs from patients without HIVD.

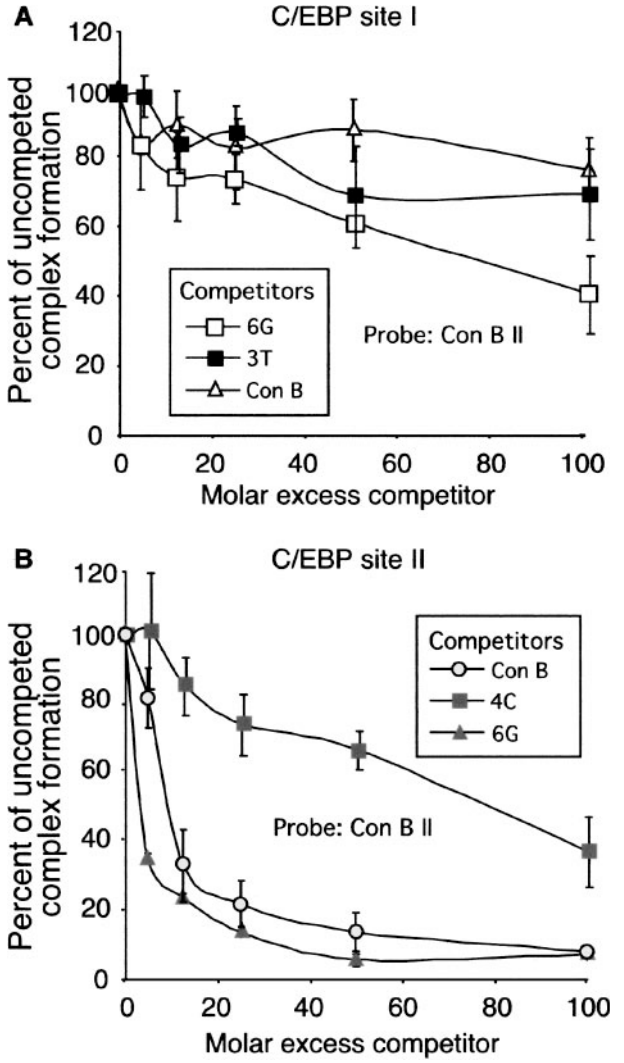

Figure 6 Consensus B and 6G C/EBP site II variants have a high affinity for C/EBP μ protein. (**A**) EMS competition analyses were performed with a labeled high-affinity consensus B site II probe and site I variants as competitors for C/EBP μ purified protein. The 6G site I had the highest affinity for C/EBP μ of all site I variants examined. (**B**) EMS competition analyses were performed with a labeled consensus B site II probe and site II variants as unlabeled competitors for C/EBP μ purified protein. Consensus B and 6G site II exhibited high affinity for C/EBP μ and 4C site II exhibited lower affinity for C/EBP μ.

complex formation by 50% (Figure 6**A**). The homologous competitor, the consensus B site II variant, required only a ninefold molar excess competitor to reduce DNA-protein complex formation by 50% (Figure 6**B**). The 6G C/EBP site II variant exhibited an even higher affinity for C/EBP μ, because it only required a threefold competitor excess to reduce complex formation by 50% (Figure 6**B**). The 4C C/EBP site II variant exhibited a low affinity for C/EBP μ, requiring 76-fold excess competitor to reduce complex formation by 50% (Figure 6**B**). In general, although 6G site I was the highest affinity C/EBP site I identified, a number of variants at site II were shown to exhibit vastly higher relative affinities for C/EBP μ than the 6G site I.

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

A series of recombinant LTR luciferase reporter constructs containing the C/EBP binding sites I and II that were shown to change in relative prevalence during the course of disease were constructed and

examined with respect to relative promoter strength in transient expression analyses. The C/EBP site I element derived from LAI (6G variant) has been shown to exhibit a moderate level of C/EBP factor recruitment, whereas the 3T site I element has been shown to exhibit comparatively weak factor recruitment (Figure 6**A**). In addition, the consensus B and 6G C/EBP site II have been shown to exhibit very strong C/EBP factor recruitment and the 4C site II has been shown to exhibit weak factor recruitment (Figure 6**B**). To begin to assess the ability of these elements to modulate basal LAI LTR activity within cells of the monocyte/macrophage lineage, transient expression analyses in the U-937 monocytic cell line were performed. This cell line was selected to facilitate comparisons of the effects of *cis*-acting C/EBP sequence variation in the cellular environment of the monocyte, which has been shown to play roles in HIV-1 invasion of the brain and the genesis of HIVD, as well as providing a potential reservoir during the course of anti-retroviral therapy.

A minimal change in basal LAI LTR activity was observed in U-937 cells when the construct containing consensus B sequence at both C/EBP sites I and II (ConB/ConB) was examined (Figure 7, ConB/ConB). Transfection of a low affinity C/EBP site I variant (3T) in the presence of a high affinity site II element (Con B) resulted in a 56% reduction in LAI LTR basal activity (Figure 7, 3T/ConB). These observations suggest that the basal LTR activity in the U-937 monocytic cell line was altered by a low affinity *cis*-acting C/EBP site I. Transient transfection of an LTR containing a low affinity C/EBP site II variant (4C) in the presence of a high affinity site I element (Con B) resulted in a 93% reduction in LAI LTR basal activity (Figure 7, ConB/4C). Examination of high affinity C/EBP site II variant (6G) in the presence of a Con B site I resulted in a 140% increase in LAI LTR activity (Figure 7, ConB/6G). These observations indicated that the basal LTR activity in the U-937 monocytic cell line was altered by a low affinity *cis*-acting C/EBP site II.

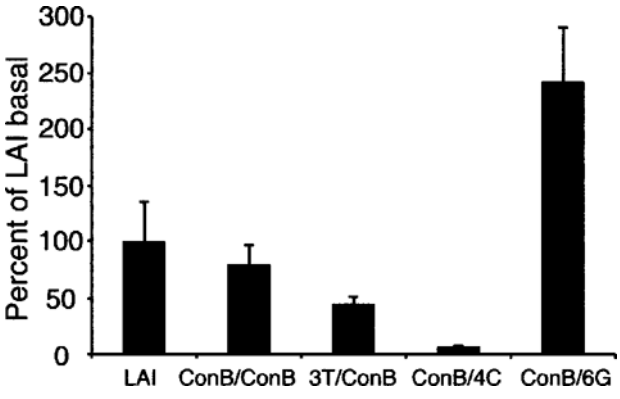

Figure 7 Impact of selected *cis*-acting C/EBP site variants on LTR activity. Transient expression analyses utilizing a dual luciferase reporter system (see Materials and methods) were employed to assess the effect of the variant C/EBP site configurations on parental LAI LTR function. Results comparing the activity of each construct in the U-937 monocytic cell line are presented as a percent of the parental LAI (6G/ConB) activity.

To examine the impact of C/EBP site variation on LTR activation in the presence of C/EBP μ, transient expression analyses were performed utilizing the pSCT-LAP C/EBP μ expression construct and variant LTR-luciferase constructs. The activities of the LAI LTR constructs containing the variant C/EBP sites were examined in the presence of increased *trans*-activating C/EBP μ in the U-937 cell line, which has been shown to express significant levels of endogenous C/EBP factors. The activities of the ConB/ConB, 3T/ConB, and ConB/4C LTR constructs all increased similarly by 2- to 2.5-fold relative to their respective basal activities (data not shown, ConB/ConB). The ConB/6G configuration, which was found at a low prevalence in later stages of disease, was unresponsive to C/EBP μ activation (0.98-fold increase over its basal level) under the conditions examined. Other C/EBP sequence variants that were present in later stages of disease were still responsive to C/EBP μ induction. This finding is relevant due to the fact that levels of IL-6, a potent inducer of C/EBP factors, are increased in the brain and peripheral blood during late stage HIV-1 infection. LTRs containing the ConB/6G configuration were not present in late stage disease. Because the ConB/6G configuration has a high basal level, viruses that contain this variant may be more prone to initiate a highly productive infection and thus may not be able to escape detection and elimination by the immune system. In addition, strains of virus with LTRs containing the ConB/6G configuration were not present in later stage disease, possibly because a high humoral immune response was effectively removing infectious particles with this configuration, precluding further infections. These strains would be more detrimental to their host. A second scenario is that viruses containing low affinity C/EBP sites can be maintained in a dormant fashion in PBMCs and then be rescued by IL-6 stimulation, inducing virus replication and consequent tissue invasion. This may account for the disappearance of the ConB/6G configuration in circulation and reappearance in CNS at late stages of disease.

Discussion

The contribution of LTR sequence variation to HIV-1 disease pathogenesis is complex. Significant evidence exists that indicates that LTR variation may alter promoter activity in different cell populations and tissue types (Henderson and Calame, 1997; Henderson *et al*, 1995, 1996; Krebs *et al*, 1998; McAllister *et al*, 2000; Quinones-Mateu *et al*, 1998; Ross *et al*, 2001a). Disease progression studies suggest that sequence variation may influence

the rapidity of pathogenesis in certain circumstances. LTR variation may play a role in tissue-specific disease, such as HIVD, or in the maintenance of viral reservoirs in particular cell populations during retroviral therapy. The ability of the LTR to function as a regulator of viral transcription is determined by the integrity of LTR sequences that bind *trans*-acting transcription factors. Viral populations that arise as a result of genomic variation during the course of disease are referred to as HIV-1 quasispecies (Goodenow *et al*, 1989), which by definition are distinct but related genomes that contain sequence changes throughout the coding and noncoding sequences, including the LTR. Numerous studies have reported sequence variation in LTRs isolated from HIV-1–infected patients (Ait-Khaled and Emery, 1994; Delassus *et al*, 1991; Michael *et al*, 1994). These studies have demonstrated both point mutations and polymorphisms within the LTR. Comparisons of LTRs derived from PBMCs and CNS also demonstrated that LTR variations are compartmentalized, and that LTRs derived from the nervous system more closely resemble the LTR from a previously characterized brain-derived HIV-1 strain (Ait- Khaled *et al*, 1995; Ross *et al*, 2001a). Changes within these LTR sequences may impact the ability of the LTR to support HIV-1 infection in different cell types by affecting binding sites for transcription factors that are either constitutive or specific to immune or nervous system cell types. The impact of LTR variation on the ability of HIV-1 quasispecies to replicate in infected cells has not yet been clearly defined. Although studies have suggested that LTR sequence variation does not lead to altered viral replication or tropism (Hirsch *et al*, 1990; Pomerantz *et al*, 1991; Schuitemaker *et al*, 1993; Velpandi *et al*, 1992), one study, using two LTR variants, demonstrated that the LTR with the higher activity in transient expression studies also directed an increase in viral replication (Golub *et al*, 1990; McAllister *et al*, 2000). LTRs derived from HIV-1–infected patients have also been shown to direct significantly different levels of transient expression within a given cell type (McAllister *et al*, 2000; Michael *et al*, 1994). Relevant to HIV-1 expression in neuroglial cells, the detection of cell type–specific reporter gene expression directed by a brain-derived LTR in transgenic mouse CNS tissue reinforces the hypothesis that LTR quasispecies are capable of cell type–specific activity (Corboy *et al*, 1992).

Based on our results regarding frequencies of C/EBP site variants in PBMCs and the brain, several inverse relationships have been observed that may be indicative of a correlation between disease severity and LTR-driven viral gene expression modulated by C/EBP factors (Figure 8**A**). At C/EBP site I, the prevalence of the consensus B C/EBP variant in PBMCs declined with increasing immunopathogenesis, whereas the frequency of its appearance in the brain was greater in patients with dementia compared to those without dementia (Figure 8**A**). In addition, the 3T site I variant was absent in LTRs derived from patients without dementia. However, 25% of LTRs derived from patients with HIVD contained the 3T C/EBP site I configuration (Figures 5**B** and 8**A**). At C/EBP site II, the prevalence of the consensus B variant increased with disease severity in PBMCs and was lower in demented patients compared to nondemented patients (Figure 8**A**). Conversely, the 6G and 4C variants of site II decreased in PBMCs with disease severity and were present at greater frequencies in brain tissue of demented patients (Figure 8**A**). These results are highly suggestive of a correlation

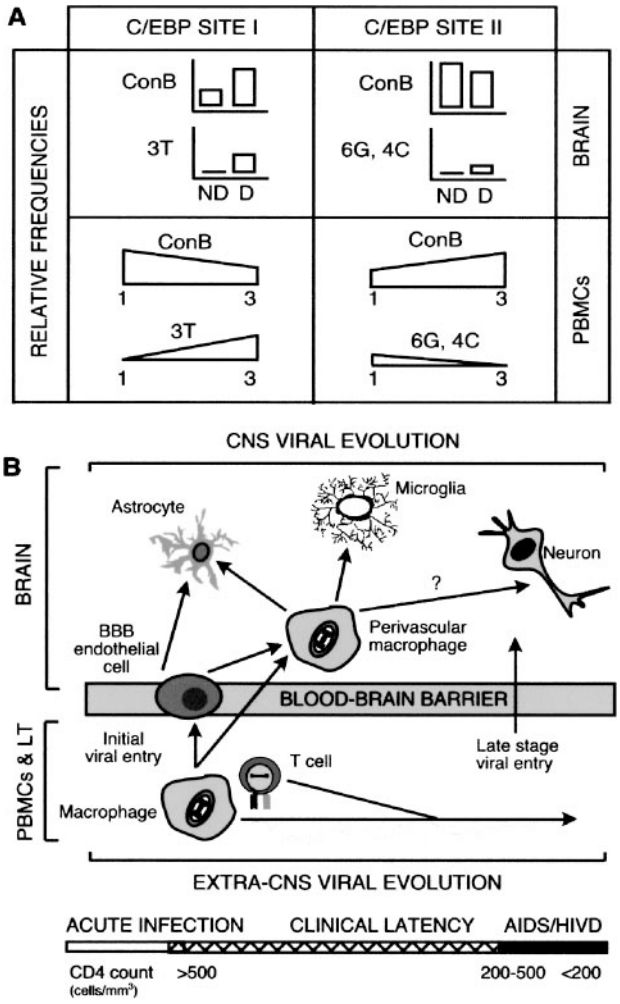

Figure 8 Model for viral evolution in PBMCs and brain during the course of HIV-1 disease. (**A**) Frequencies of sequence variants were compared at C/EBP site I and C/EBP site II in PBMCs and brain during the course of disease. The triangle represents an increase or decrease in the prevalence of the indicated variant during disease progression from DSG 1 to DSG 3. ND, LTRs derived from patients without HIVD; D, LTRs derived from patients with HIVD. (**B**) Model for HIV-1 evolution in PBMCs and the brain. Viral evolution can occur in the brain after the initial viral entry or evolve in the blood and then enter the brain during late stage disease. (This does not preclude viral-specific evolution within the CNS compartment during the course of disease.)

between C/EBP site variation, LTR function, and disease progression in the peripheral blood and brain.

During the course of disease, the cells of the monocyte/macrophage lineage may carry virus across the blood-brain barrier where virus can infect and/or cause dysfunction of perivascular macrophages, astrocytes, microglia, and possibly neurons during primary disease or during the later stages of disease (Figure 8**B**). Viral quasispecies that ultimately cause CNS disease and HIVD may evolve in the CNS after initial viral entry or in extra-CNS compartments, such as PBMCs, lymph nodes, or bone marrow, and then migrate to the brain in the later stages of disease, resulting in dysregulation of brain function and HIVD (Figure 8**B**). These results suggest that evolution of the HIV-1 LTR, specifically C/EBP sites I and II, occurs in both the brain and peripheral blood and that at least a subset of the evolutionary changes could be compartment-specific and may very well be cell type-specific. Another possibility is migration of the infected PBMCs in the brain rather than divergent evolution in separate compartments. Although PBMCs can harbor highly replication competent viruses (Asjo *et al*, 1986), this cell population may also harbor viruses with low replication potential or viruses that are conditionally defective and may become replication proficient with a change in the intracellular environment, such as a change in IL-6 levels. Activation of HIV-1–infected PBMC-derived cells may lead to enhanced trafficking of these cells to the brain. This could be the case with the 4C and 6G C/EBP site II variants because their frequencies decreased in PBMCs with increasing disease severity and were found at higher levels in brain-derived LTRs from patients with HIVD than those without HIVD. The increase in LTRs containing consensus B site II in PBMCs over time could be due to the loss of this type of LTRs to specific tissues. In addition, at end-stage disease, the PBMC population contains mostly cells of the monocyte/macrophage lineage, because the T-cell population was depleted and the monocytic cells are the cells responsible for carrying viruses into the brain.

The 3T variant of C/EBP site I was present in 48% of LTRs in late stage disease in peripheral blood and in 25% of HIV-1–demented brains. This may be linked to the low relative affinity for C/EBP μ (Figure 6**A**) and the high relative affinity for Vpr (Hogan *et al*, 2003). As HIV-1 disease progresses, viral quasispecies in the peripheral blood evolve with LTRs containing a high-affinity site relative to Vpr binding to potentially facilitate Vpr-mediated LTR activation and viral gene expression. HIV-1 LTR C/EBP 3T site I variants with high relative affinity for Vpr were prevalent in both peripheral blood and brain tissue of viral LTRs obtained at late stage disease (Hogan *et al*, 2003). This suggests an important role for Vpr in the progression of HIV-1 infection and specifically the genesis of HIVD. Furthermore, the increase in prevalence of the C/EBP site I 3T variant with increasing disease severity in both the peripheral blood and brain indicates that this site may serve as an important molecular marker for disease progression in both the peripheral immune system and CNS.

Materials and methods

Polymerase chain reaction (PCR) amplification of brain tissue

Freshly autopsied brain specimens from HIV-1–infected patients with and without clinical dementia were collected with informed consent 4 to 36 h postmortem. Prior to homogenization, the meninges were removed and the brain tissues were washed in Dulbecco's phosphate-buffered saline (PBS) without magnesium and calcium to remove contaminating blood. High-molecular-weight DNA was purified from fresh uncultured brain tissue by phenol/chloroform extraction. A fragment of approximately 970 bp, which included the HIV *nef* gene and the 3′ LTR was amplified from the brain tissue using PCR. In some cases, nested PCR was utilized to obtain a signal that was sufficient for molecular cloning. Approximately 1 μg of genomic DNA was used in the first round of amplification, and when nested PCR was used, 1 μl of the first round reaction was used for the second round. The outer primers used for PCR amplification were 5′-CGAAGGAATAGAAGAAGAAGGTG and 5′-CAGTGTGGAAAATCTCTAGC. The inner primers used were 5′-CCTAGAAGAATAAGACAGGGCTT and 5′-GGTCTGAGGGATTGTAGTTAC. Amplification consisted of 30 cycles of 60 s at 94≈C, 60 s at 53≈C, and 3 min at 72≈C. Amplified products were ligated into the pCR2.1 vector (Invitrogen, Carlsbad, CA) and the ligation reactions used to transform STBL2 bacterial cells (Gibco BRL, Carlsbad, CA). Clones were screened by hybridization and confirmed for size by EcoR1 digestion. Automated sequencing was performed using the dye terminator method and either the ABI 373 and 377 instrument. Sequence results were edited and analyzed using the DNAstar software package.

Statistical analyses

Chi-square analysis was performed to determine if there was an association between severity of disease and sequence variation in PBMCs at C/EBP sites I and II (Figures 2 and 3). The Fisher's exact test was performed to determine if there was an association between severity of disease and sequence variation in PBMCs at NF-μB site II (Figure 4). Chi-square analysis was performed to determine if there was an association between severity of disease and sequence variation in brain at C/EBP site I and II (Figure 5**A** and **B**).

Cell culture
The U-937 human monocytic cell line (ATCC CRL-1593.2) was grown in RPMI 1640 medium 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.

Oligonucleotide synthesis and radiolabeling
Complementary single-stranded oligonucleotides corresponding to the published C/EBP sequences within the HIV-1 LTR 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 [μ-^{32}P]-ATP and T4 polynucleotide kinase as described (Promega, Madison, WI). The sequences of the probes used in these studies were as follows: Con B site I: TCG AGC TTT CTA CAA GGG; 6G site I: TCG AGC TTG CTA CAA GGG; 3T site I: TCG AGT TTT CTA CAA GGG; Con B site II: CTA GCA TTT CAT CAC GTG; 6G site II: CTA GCA TTT CGT CAC GTG and 4C site II: CTA GCA TTC CAT CAC GTG. Sequences flanking the C/EBP binding sites were those present in the LAI LTR. The C/EBP binding sites are indicated as underlined sequences.

Electrophoretic mobility shift (EMS) analyses
EMS binding reactions were performed. Briefly, radiolabeled probe (C/EBP consensus B site II) and poly [d(I-C)] (1 μg) were incubated with purified C/EBP μ-His (600 ng) for 30 min at 30°C. Reactions (15 μl, final volume) were supplemented with bovine serum albumin (BSA) (15 μg). Competition EMS analyses were conducted by adding the indicated molar excess of unlabeled homologous or heterologous oligonucleotide probe. DNA-protein complexes were resolved by electrophoresis for 2 h at 30 mA and 4°C in a 5% nondenaturing polyacrylamide gel, which was subsequently dried at 80°C for 1 h, and subjected to autoradiography.

Plasmids and site-directed mutagenesis
The luciferase reporter construct containing the LTR derived from the HIV-1 LAI molecular clone was constructed as described (McAllister *et al*, 2000). The parental LAI luciferase construct was then used as a template for site-directed mutagenesis. The QuickChange mutagenesis kit (Stratagene, La Jolla, CA) was used to construct ConB/ConB, 3T/ConB, ConB/4C, and ConB/6G (C/EBP site I/C/EBP site II) plasmids. 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).

Protein purification
A polyhistidine-tagged C/EBP μ construct (C/EBP μ-BD-pRSET A) was obtained from Dr. Edward Maytin (Lerner Research Institute, Cleveland, OH). This plasmid was transformed into *Escherichia coli* strain BL21 (DE3) pLysS; transformants were selected using ampicillin (50 μg/ml) and chloramphenicol (35 μg/ml). Expression of the 6$\approx$ histidine-tagged protein was induced for 5 h with 1 mM isopropyl μ-D-thiogalactoside (IPTG) in 1$\approx$ YT medium. C/EBP μ was then purified on nickel-chelating columns using imidazole elution (pRSET Xpress, Invitrogen). Protein integrity and purity was assessed by Western blot analysis and silver staining (Bronstein *et al*, 1992; Burnette, 1981; Gillespie and Hudspeth, 1991; Sandhu *et al*, 1991).

Transient expression analyses
Exponentially growing U-937 cells were seeded at 4 $\approx$ 10^5 cells in 500 μl growth medium without antibiotics and transfected with Lipofectamine 2000 (Invitrogen) in 24-well plates (TPP, Switzerland). After a series of optimization experiments were performed in which U-937 cells were transfected with varying amounts of Lipofectamine 2000 and pGFP DNA to determine peak transfection efficiency (5 μl Lipofectamine 2000 with 0.8 μg of DNA), premixed Lipofectamine 2000 (45 μl reduced serum Opti-MEM with 5 μl Lipofectamine) was added to 0.75 μg of the luciferase vector and 50 ng pRL-TK Renilla internal control vector diluted in 50 μl Opti-MEM. When cotransfection with C/EBP μ was performed, the additional expression constructs were included in the lipofectamine-DNA mixture. The C/EBP μ expression construct (pSCT-LAP) was added at 10% the level of the experimental LTR-luciferase vector. The C/EBP μ expression vector was provided by Dr. Andrew Henderson (The Pennsylvania State University, University Park, PA). After a 20-min incubation, the Lipofectamine-DNA mixture was added directly to the cells. Cultures were incubated at 37°C in 5% CO_2 for 24 h. Cell extracts were harvested and assayed for luminescence using a Fluoroskan Ascent FL (Thermo Labsystems, Franklin, MA) as described for the Dual Luciferase Assay (Promega). Firefly luminescence was normalized to the Renilla luminescence to control for variability in transfection efficiency. Firefly luminescence (pGL3 constructs) was presented with the parental activity set to 1.0 for each experiment and the relative activity of the mutagenized constructs calculated accordingly. Error bars in figures indicate the standard deviation. Each value shown represents the average of three independent experiments performed with triplicate samples.

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