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HIV-1 LTR DNA sequence variation in brain-derived isolates

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Isolates of human immunodeficiency virus (HIV-1) derived from the central nervous system (CNS) display properties distinctive from blood-derived isolates, including a high incidence of macrophage tropism in CNS isolates. Macrophage tropism is a result, in part, of DNA sequence variation in the HIV-1 envelope glycoprotein gene, but evidence also exists suggesting differences in the long terminal repeat (LTR) may contribute to differential gene expression. To investigate the nature of HIV-1 LTR sequence variation in the brain, we have sequenced bases −374 to +43 of the LTR from the brains of four HIV-1-infected patients. A total of 56 clones were derived from either both gray and white matter (three brains) or white matter alone (one brain), and these sequences were compared to 17 published sequences derived from multiple sources. A total of five LTR quasispecies were found. Overall, there was a significant amount of sequence variation both within and between brains, comparable to that seen in quasispecies of the envelope glycoprotein derived from blood or brain. The vast majority of the variation was seen in regions upstream from the two NF-κB sites. Compared to the blood-derived, T cell-tropic IIIB LTR, a majority of clones from two or more of the brains shared 11 unique substitutions in transcription factor binding sites, of which eight were shared with the CNS-derived clones JR-CSF and JR-FL and altered the NF-AT and LEF-1 transcription factor binding sites. These findings correlate with published functional studies showing CNS-derived HIV-1 LTRs are distinct from the blood-derived IIIB LTR, and represent a starting point for future studies designed to determine which LTR sequence variations are associated with cell-specific differences in gene expression in the CNS.

Keywords: HIV-1; LTR; brain; AIDS dementia complex; transcription factor binding sites; viral tissue tropism

Introduction

The human immunodeficiency virus (HIV-1) invades the central nervous system (CNS) early after infection (Davis *et al*, 1992; Resnick *et al*, 1988), causing a slowly progressive dementia, the acquired immunodeficiency syndrome (AIDS) dementia complex (ADC), in 30–60% of patients (Atwood *et al*, 1993; McArthur *et al*, 1994; Navia *et al*, 1986). The relationship between intraparenchymal HIV-1 viral load, neuropsychological disease and HIV-1 associated neuropathological changes remains to be determined, but the available evidence supports the notion that HIV-1 viral load in the brain is associated with the clinical and neuropathological changes seen in AIDS patients (Achim *et al*, 1994; Böni *et al*, 1993; Brüstle *et al*, 1992; Epstein *et al*, 1987; Glass *et al*, 1995; Johnson *et al*, 1996; Masliah *et al*, 1992; Pang *et al*, 1991; Weiser *et al*, 1990; Wiley *et al*, 1996). Isolates of HIV-1 from the CNS display properties which distinguish them from peripheral blood isolates, such as relative inability to infect T lymphocyte cell lines, efficient replication in macrophages and microglia, and reduced cytopathogenicity (Anand *et al*, 1987; Cheng-Mayer *et al*, 1989), suggesting CNS isolates may represent a separate subgroup of HIV-1. The regions of the HIV-1 viral genome important for CNS tissue tropism and disease potential have not been completely defined. The cells in the CNS most commonly found to be infected by HIV-1 are macrophages and microglia (Koenig *et al*, 1986; Wiley *et al*, 1986), and most of the quasispecies derived from the central nervous system (CNS) display macrophage tropism (Cheng-Mayer *et al*, 1990; Cheng-Mayer *et al*, 1989; Korber *et al*, 1994;

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Koyanagi *et al*, 1987; Liu *et al*, 1990; O'Brien *et al*, 1990; Sharpless *et al*, 1992; Watkins *et al*, 1990). Macrophage tropism has been ascribed to DNA sequence variations in the V3 loop and possibly other regions of the envelope glycoprotein, gp120 (Boyd *et al*, 1993; Cann *et al*, 1992; Cheng-Mayer *et al*, 1990; Chesebro *et al*, 1992; Groenink *et al*, 1992; Hwang *et al*, 1991; Korber *et al*, 1994; Liu *et al*, 1990; O'Brien *et al*, 1990; Sharpless *et al*, 1992; Shimizu *et al*, 1994; Shioda *et al*, 1991), encoded by the *env* gene. Although there have been reports suggesting signature amino acid sequences exist in HIV-1 gp120 variants in the CNS (Korber *et al*, 1994), or that one or more amino acid substitutions in the V3 loop discriminate between patients with and without ADC (Power *et al*, 1994), these findings have not been reproduced (Kuiken *et al*, 1995).

The long terminal repeat (LTR), where the promoter and enhancer regions of the virus are located, might also contribute to tissue tropism. As with the HIV-1 *env* gene (Ball *et al*, 1994; Epstein *et al*, 1991; Korber *et al*, 1994; Pang *et al*, 1991), HIV-1 LTR DNA sequences appear to evolve independently in the CNS (Ait-Khaled *et al*, 1995). In other retroviruses, variant LTR sequences have been associated with tissue tropism and disease potential (Celander and Haseltine, 1984; Lenz *et al*, 1984; Rosen *et al*, 1985; Small *et al*, 1989), sometimes in association with the *env* gene (Poliquin *et al*, 1992; Portis *et al*, 1990; Yuen *et al*, 1986). Some groups have found cell type-specific differences in replication or gene expression in association with various substitutions, additions or deletions within the HIV-1 LTR (Delassus *et al*, 1991; Golub *et al*, 1990; Kim *et al*, 1993; Koken *et al*, 1992; Michael *et al*, 1994; Tillman *et al*, 1994; Zeichner *et al*, 1992), while others have not (Pomerantz *et al*, 1991). We previously showed the LTRs of strains JR-CSF and JR-FL, derived from the cerebrospinal fluid (CSF) and frontal lobe, respectively, of a patient with ADC (Koyanagi *et al*, 1987), are able to direct the expression of a reporter gene in the CNS of transgenic mice, whereas the blood-derived, T cell-tropic strain HIV IIIB LTR is not (Corboy *et al*, 1992). Subsequent studies of *in utero*, neonatal and adult mice constructed with the JR-FL and JR-CSF LTRs revealed CNS expression in these transgenic mice varies by location and cell type over time (Buzy *et al*, 1995), which may be relevant to the clinical and neuropathological differences seen between adult and pediatric HIV-1 patients (Sharer, 1992).

Recent studies have attempted to determine which regions within the LTR might contribute to tissue- and cell-specific gene expression within the CNS. Using DNA footprint analysis and electrophoretic mobility shift assays (EMSAs), Cannone-Hergaux *et al* (1995) found nuclear extracts from cultured astrocytic (U-373 MG), neuronal (SK-N-MC) and T lymphocytic cells (Jurkat) bound with different affinities the JR-FL, JR-CSF and LAI (IIIB) LTRs in the region −352 to −324. Further, variant sequences encompassing an AP1 site in both neurotropic strains, in the region −247 to −222, could bind U-373 MG extracts, but not those from SK-N-MC or Jurkat cells, and this variant sequence was able to mediate AP1-induced transcriptional activation in U-373 MG but not SK-N-MC or Jurkat cells. In contrast, Kurth *et al* (1996) detected binding in the region −300 to −260 using an *in vivo* DNA footprinting analysis with brain extracts of transgenic mice expressing JR-CSF, and brain extracts of nontransgenic newborn mice were able to bind region −296 to −256 of JR-CSF but not IIIB LTR. Finally, studies with linker scanning mutants of the HIV-1 LTR transfected into the neuron-like NTERA cells have revealed two upstream regions (−219 to −202 and −255 to −238) important for cell-specific gene expression (Zeichner *et al*, 1992).

Thus, HIV-1 LTR quasispecies may evolve independently within the CNS, and may be associated with cell- and tissue-specific nuclear extract binding and gene expression. Which regions of the LTR have greatest importance in the CNS remain unclear. In order to define further the spectrum of sequences potentially relevant to HIV-1 gene expression in the CNS, we analyzed HIV-1 LTR DNA isolated from the brains of four HIV-1-infected adult patients. The sequences were aligned and compared by phylogenetic analysis to themselves (intra-brain and inter-brain), the CNS-derived clones described by Ait-Khaled *et al* (1995), and to 16 previously published LTR sequences deposited in the Los Alamos HIV-1 sequence databank (Myers *et al*, 1995).

Results

Comparisons within brains, between brains and to published clones

DNA was amplified, cloned and sequenced from a total of seven sections of four brains: from gray and white matter of three brains, and white matter alone of one brain. All 56 sequences fit into clade B of sequences entered in the Los Alamos AIDS sequence databank (Myers *et al*, 1995). A sequence homology and phylogenetic analysis of bases −374 to +43 of the LTR revealed a range of 85.4–100% sequence similarity within any one brain, with the weighted mean of 96.4%. Three of the brains (2627, 2297, 2135) had two distinct quasispecies, and one (2444) had one, or possibly two. Two of the three brains from which both gray and white matter clones were sequenced (2135, 2627) had a quasispecies common to both gray and white matter, and a second quasispecies seen only in the white (2627) or gray (2135) matter. The third brain (2444) had a gradient of sequence similarity, with the white and gray matter sub-

groups in group B (Figure 1) possibly representing separate quasispecies. The brain represented by white matter clones alone (2297) displayed two quasispecies, one of which was shared with brain 2135. Sequence similarity between clones from different brains ranged from 85.2–100% per clone, with a mean of 92.8%. Thus, overall there were at least five distinct populations of sequences, or, quasispecies (A-E), as seen in Figure 1. The 16 comparable LTR sequences (Figure 1) in clade B of the Los Alamos HIV-1 sequence database (Myers *et al*, 1995), derived from multiple tissues and sources, shared 93% similarity with one another, and with our brain clones. When placed on our phylogenetic tree, the sequences described by Ait-Khaled *et al* (1995), derived from spinal cord and dorsal root ganglia of a single patient, occupied a branch (group F) separate from our clones and equally distant from IIIB, JR-FL and JR-CSF.

Variation within transcription factor binding sites

A simplified scheme of agreed-upon transcription factor binding sites within the HIV-1 LTR (Gaynor, 1992) is shown in Figure 2. These sites encompass 55% of all bases within the range of amplified LTR in these sequences. Of the 120 positions at which any of our brain clones differed from the consensus of the brain clones, 65 (54%) occurred within transcription factor binding sites. This was true whether the substitution was rare or common. Substitutions, additions or deletions were rare, however, within the three Sp1 sites and the two NF-κB sites, and the brain consensus sequence was identical to the clade B consensus in these areas. Similarly, there were few alterations from the clade B consensus in the TATA region, UBP regions, or in that portion of the TAR region we amplified. There was much greater diversity within the NF-AT, LEF-1 COUP, and USF transcription factor binding sites, in comparison to the brain consensus, the clade B consensus, and IIIB (Figure 2).

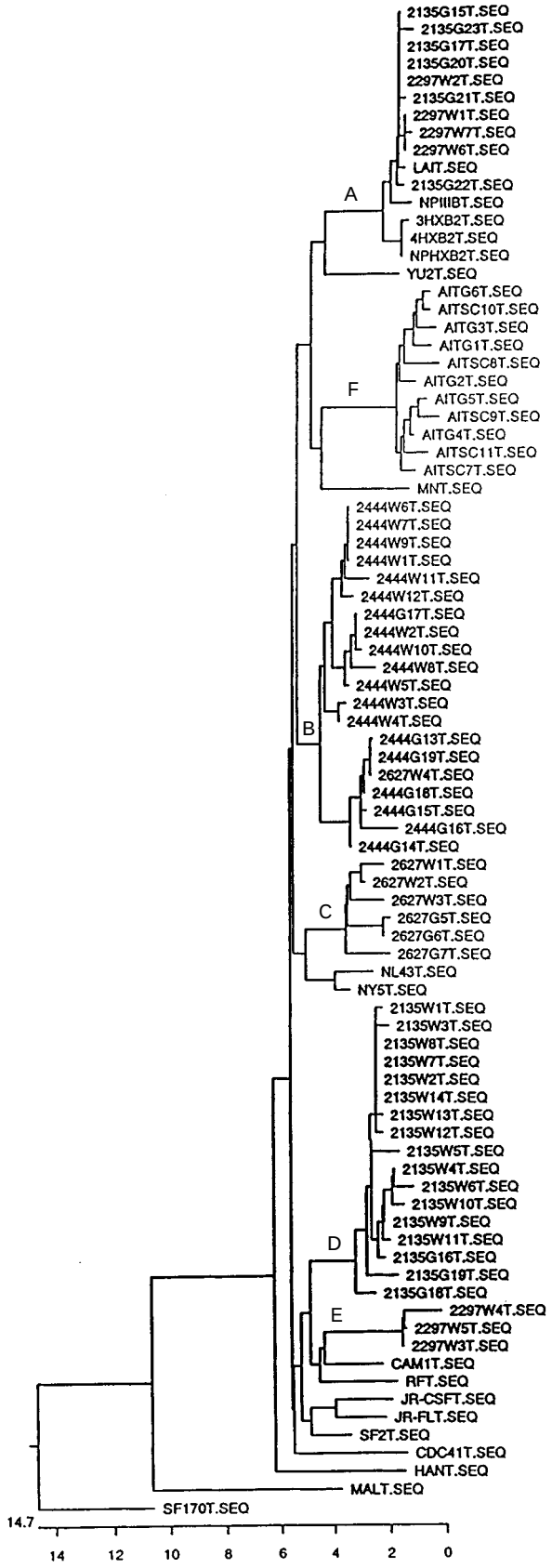

Figure 1 Phylogenetic tree of brain-derived HIV-1 LTRs. Phylogenetic tree comparing bases −374 to +43 of the HIV-1 LTR in 56 brain-derived clones to spinal cord and dorsal root ganglia clones from Ait-Khaled *et al* (1995), and to IIIB, HXB2, JR-CSF, JR-FL, CAM, CD41, HAN, LAI, MAL, MN, NL43, NY5, RF, SF170, SF2 and YU2 (Myers *et al*, 1995). The 'T' at the end of each sequence denotes that these are sequences truncated to include only bases −374 to +43. 'NP' before the IIIB and HXB2 sequences denote that these sequences are taken from DNA which was *not* PCR-amplified. '3HXB2' and '4HXB2' are two clones which *were* PCR-amplified from plasmid DNA, and which are identical to the 'NPHXB2'. This unrooted, unbalanced tree is obtained using the neighbor-joining method (Saitou and Nei, 1987). The length of each pair of branches represents the distance between sequence pairs. The scale beneath the tree measures the distance between sequences, and the units indicate the number of substitution events. The capital letters A-F denote the presumed quasispecies.

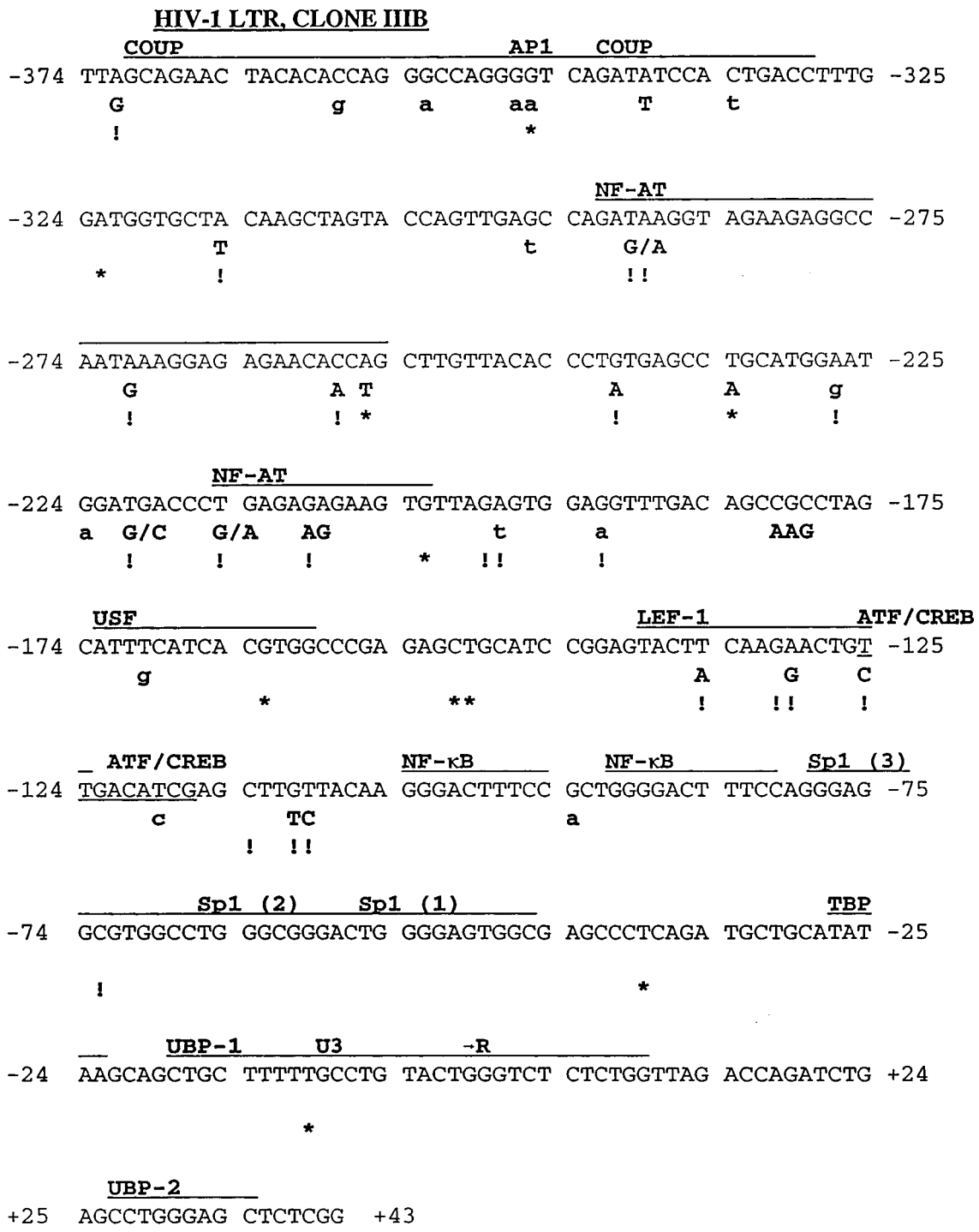

Figure 2 HIV-1 LTR, clone IIIB. Bases −374 to +43 of clone IIIB LTR, with agreed upon trascription factors (Gaynor, 1992) shown above the DNA motifs to which they bind (note overlap of LEF-1 and ATF/CREB sites). Sites at which both JR-FL and JR-CSF differ from IIIB (JR substitution sites) are noted in bold, upper-case letters below the sequence, with lower-case letters representing substitutions in either JR-CSF or JR-FL. Also marked below the IIIB sequence are those positions at which 14 or more (*) or 28 or more (!) of our 56 brain clones (in at least two of the four brains) differ from IIIB. Substitutions in our brain clones at JR substitution sites are identical to the JR substitutions. The transcription factor binding sites are indicated by the first letter of the name at the 5′ end, and the end of the underline at the 3′ end. Abbreviations: COUP, Chicken Ovalbumin Upstream Promoter; AP1, Activating Protein 1; NF-AT, Nuclear Factor of Activated T cells; USF, Upstream Stimulatory Factor; LEF-1, Lymphocyte Enhancer Factor-1; ATF/CREB, Activating Transcription Factor/cAMP Response Element Binding; NF-κB, Nuclear Factor-κB; Sp1, SV40 Promoter 1; TBP, TATA Binding Protein; UBP, Untranslated Binding Protein. Note that not all DNA motifs conform to consensus descriptions, as some HIV-1 clones are variable in these regions.

We have already shown HIV-1 IIIB LTR does not, while the CNS-derived clones JR-FL and JR-CSF do, drive expression of a reporter gene in the CNS of transgenic mice (Corboy *et al*, 1992). This functional difference presumably is related to differences in the DNA sequences of the LTRs. From bases −374 to +43 of the LTR, there are 21 base pair differences distinguishing IIIB from both JR-FL and JR-CSF (Figure 2). These differences are referred to here as JR substitutions. Clones in group A (Figure 1) shared 4-7 JR substitutions, while sequences in group B-E shared 10-17 JR substitutions.

Eleven of 21 (52%) JR substitutions fell within transcription factor binding sites, seven within the two designated NF-AT motifs (positions −292 to −255 and −215 to −203), three within the LEF-1 site (−139 to −124) and one within the COUP site (Figure 2). Thus, although the NF-AT transcription factor binding sites constitute just 12% of the total amplified LTR 33% of all JR substitutions occurred within these sites. At eight of the 11 JR substitution locations within transcription factor binding sites, including five in the NF-AT and three in LEF-1 binding sites, a majority of our brain clones shared the exact JR substitution (Figure 2).

There also were six JR substitution sites outside of the agreed-upon transcription factor binding sites at which a majority of our brain clones shared the same JR substitution. Overall, at a total of 14 JR substitution sites, a majority of our brain clones were identical to the JR substitution. Among the published sequences noted in Figure 1 (total of 14, excluding HXB2 and the two JR clones, and using the consensus of the clones from {2}), the number of JR substitutions shared was as few as four (LAI) and many as 19 (SF2), with a mean of 13.8. On average, 8.6 of the 14 clones compared above shared any one JR substitution. There was no correleation of macrophage-tropic or T cell-tropic strains of HIV-1 with number of JR substitutions in that clone.

In addition to JR substitutions shared by a majority of our clones, there also were eight other locations at which a majority of our brain-derived clones differed from IIIB. At three of these locations, either JR-FL or JR-CSF differed from IIIB, and at five both JR clones were the same as IIIB. Just three of these other locations were within transcription factor binding sites (NF-AT, LEF-1 and Sp1), but three occurred very near one another, between bases −198 and −192. Therefore, there were a total of 11 locations within transcription factor binding sites at which a majority of our clones differed from IIIB, eight of which were shared by the JR clones. Of the 14 comparison clones noted above, an average of 9.3 of these clones also shared the substitutions that distinguished our clones from IIIB.

Multiplication of ATF/CRE transcription factor binding sites

Recently, an ATF/CREB site was identified just upstream from the two NF-κB sites in the HIV-1 LTR derived from multiple blood samples, and was shown to bind ATF/CREB factors derived from U-373 MG astrocytic cells (Krebs *et al*, 1997). Analysis of our brain-derived sequences, all 16 of the comparable clones in the Los Alamos HIV-1 sequence database (45-Figure 1), and the clones described by Ait-Khaled *et al* (1995)

ATF/CREB DUPLICATIONS

```
-139                                                        -117
TACTACAAGGACT------GTTGACATCGAG--TGCTGAGACTGCTGACATCG   CONSENSUS
        A    AAGACT C     A A  -- T CATA                  94299
 T      A    ------           AC                         2297W3
 T      A    ------         -  AC                         2297W4
 T      A    ------           AC                         2297W5
             ------------------------------       C     JR-CSF
             ------------------------------             JR-FL
    T    A   ------------------------------             IIIB

LEF-1
                                                ATF/CREB
```

Figure 3 ATF/CREB duplications. Three brain-derived and one blood-derived (94299) sequences are aligned with bases −139 to −117 of IIIB, JR-FL, and JR-CSF, revealing 23–28 bp insertions creating one or two extra putative ATF/CRE transcription factor binding sites (CTGACAT/CCG, Krebs, *et al*, unpulbished data). These insertions interrupt the LEF-1 site. The consensus sequence represents the majority bases of the seven sequences listed. The extra six bases at the 5′ end of clone 94299 recapitulates, in part, the LEF-1 site. The two putative ATF/CRE duplications are underlined in the consensus sequence.

and Estable *et al* (1996) revealed little overall variation within this ATF/CREB site. In brain sample 2297, we sequenced three clones with an apparent extra two ATF/CREB sites embedded in 23–24 bp insertions at position −126 (Figure 3). These insertions interrupted the LEF-1 site, and were similar in location and DNA sequence to the LEF-1 duplications described in blood samples by others (Ait-Khaled *et al*, 1995; Delassus *et al*, 1991; Estable *et al*, 1996; Golub *et al*, 1990; Michael *et al*, 1994). The insertions differed from each other at only a single base, and shared 15 bases with the 28 bp insertion in clone, 94299, derived from the blood of an unrelated patient (Figure 3).

Discussion

In this study, we describe the amount and the location of DNA sequence variation in HIV-1 LTR sequences derived from the brains of four HIV-1-infected patients. The degree of sequence variation in the *env* region in brain samples has varied widely, from as little as 2.1% differences between brains (Monken *et al*, 1995) to a maximum of 11.8% (Korber *et al*, 1994). Variation in *env* may be somewhat less in brain than blood samples, suggesting either a lack of divergence or a convergence to macrophage-tropism among HIV-1 *env* sequences derived from the brain (Korber *et al*, 1994). Within a single brain, Korber *et al* (1994) found significantly fewer sequence differences in the *env* region with respect to comparisons made between brains (3.5% *vs* 11.8%). In our brain samples we found 96.4% sequence similarity (3.6% difference) within a brain sample and 92.8% (7.2% difference) between brain samples. Thus, the amount of sequence variation in our brain-derived LTR samples is comparable to the variation in brain-derived *env* sequences.

In this study, we can not determine whether any particular HIV-1 LTR sequence is associated with ADC or other neurological syndromes. It is interesting to note, however, the patient described by Ait-Khaled *et al* (1995) had vacuolar myelopathy without ADC, and the clones derived from the spinal cord and dorsal root ganglia of this patient were quite different from our brain-derived clones, although they shared 15 JR substitutions. Thus, there may be variation in the HIV-1 LTR which is relevant to location-specific viral replication within the CNS. It also is possible that had Ait-Khaled *et al* (1995) obtained sequences from more than one patient, there might have been greater overlap with our brain-derived clones.

The substitutions in the LTR described here, whether in comparison to the consensus of all the brain sequences or to the clade B consensus, were not random. Similar to other reports of brain-derived (Pomerantz *et al*, 1991) and some blood-derived LTR sequences (Estable *et al*, 1996; Krebs *et al*, 1997), we found very few alterations in both NF-κB sites, two of three Sp1 sites, the ATF/CREB sites and the TAR regions, suggesting these sites are necessary for transcription to function properly in the brain. Others, looking primarily at clones derived from blood or peripheral blood mononuclear cells, have identified more significant changes within the NF-κB, SP-1 and TAR bindings sites. (Ait-Khaled *et al*, 1995; Delassus *et al*, 1991; Koken *et al*, 1992; McNearney *et al*, 1995).

The majority of variation in our brain samples was upstream of the second NF-κB binding site, many of the changes altering transcription factor binding sites in what has been termed the negative regulatory element (NRE). The NRE encompasses the NF-AT and USF binding sites, and has potential repressor and activator functions (Gaynor, 1992; Lu *et al*, 1990; Orchard *et al*, 1990; Rosen *et al*, 1985). A disproportionate number of JR substitutions occurred within the two NF-AT binding sites (especially the upstream site at nucleotides −292 to −255), and many of our brain clones and other published clones from the CNS (Ait-Khaled *et al*, 1995; Li *et al*, 1992) and other tissues shared these substitutions. The JR substitutions changed the NF-AT binding site from −292 to −255 at multiple positions, potentially altering transcription significantly in different cells. Further suggesting the importance of this region were the recent findings of Kurth *et al* (1996), in which the region −296 to −256 of the JR-CSF LTR was able to bind brain-derived nuclear proteins, while the highly T cell-tropic IIIB LTR in the same region was not. The transfection studies of Zeichner *et al* (1992), show sequence variation at the other NF-AT site, −219 to −202, a region in which many of our clones share JR substitutions, is associated with differential gene expression in the neuron-like NTERA cells, but binding studies have not yet shown this area to be important in neural cells.

Changes in the LEF-1 transcription factor binding site have the same potential for affecting transcription. Six of the sites at which a majority of our brain-derived LTR clones differed from IIB fell between positions −201 and −130, a region in which DNA substitutions have been associated with significantly lowered transcriptional activity in T cells (Kim *et al*, 1993). This confluence of brain-derived LTR sequences sharing specific substitutions within the NF-AT, USF and LEF-1 sites strongly suggests these changes result in altered HIV-1 gene expression in T cells, and may, in part, explain why many brain-derived clones are macrophage-tropic.

In a related study (Krebs *et al*, unpublished data) using electrophoretic mobility shift assays, we have analyzed the effect that multimerization of the ATF/CREB site has on the ability to bind nuclear extracts from cells of astrocytic (U-373 MG) and T lympho-

cyte (Jurkat) origin. The oligonucleotide probe representing the multimerized ATF/CREB site in clone 2297w3 (Figure 3), when reacted with nuclear extract from Jurkat cells, produced DNA-protein complexes which were different in abundance and mobility compared to complexes produced with the same probe but nuclear extracts from the U-373 MG cells (data not shown). The differences in complex formation between these two cell types suggest differences in factor recruitment and site occupation, and provide another mechanism by which different LTR quasispecies may produce functional alterations in transcription capabilities in different cells in the CNS and elsewhere.

In comparing a number of published LTR sequences to IIIB, the JR clones and our brain-derived clones, it is clear clones derived from a number of sources share varying amounts of sequence similarity with our brain-derived clones. There is no simple correlation, however, between sharing a large number of JR substitutions and T cell-tropism or macrophage-tropism. For example, clone SF2, initially obtained by cocultivation from peripheral blood mononuclear cells, is T cell-tropic (Cheng-Mayer *et al*, 1989) yet shares 19 of 21 JR substitutions in the LTR. This suggests several possibilities. First, with the exception of disturbances in the core promoter and enhancer regions (e.g. TATA, Sp1 and NF-κB sites), variation in the LTR might be meaningless, especially with regard to differential gene expression within the brain. The functional data described above strongly argues against this. Second, some LTR sequences may represent clones which recently entered the CNS, while others might represent species which have been present in the CNS for a longer period of time, and which have evolved independently from the blood, as suggested by Ait-Khaled *et al* (1995). Without blood samples from the same patients, we can not answer this question directly. The third possibility is HIV-1 infects multiple different cells in the brain, and viral strains display a range of abilities to replicate within the various cells. The cells in the CNS which most commonly are found to be productively infected by HIV-1 are macrophages and microglia (Koenig *et al*, 1986; Wiley *et al*, 1996), explaining why most CNS-derived strains of HIV-1 are macrophage-tropic. But recent evidence shows astrocytes (Saito *et al*, 1994; Takahashi *et al*, 1996; Tornatore *et al*, 1994) and neurons (Bagasra *et al*, 1996; Nuovo *et al*, 1994) also are infected, albeit at a lower level. Thus it is possible that CNS-derived isolates of HIV-1 may have tropism for one or more of several cell types. Changes in the LTR could be associated with differences in transcriptional function in different cells, which might, in part, account for the relative difficulty in obtaining evidence of HIV-1 infection in neural cells. This also might explain why some HIV-1 isolates obtained from blood (Cheng-Mayer *et al*, 1989) or CSF (Koyanagi *et al*, 1987) are T cell-tropic, yet are relatively more able to infect cells of astrocytic origin than are macrophage-tropic isolates. Future studies with our brain-derived clones will address questions regarding the nature of virus-host cell interaction in cells relevant to the CNS.

Table 1 HIV-1 LTR primers for PCR and DNA sequencing primer

Name		*Primer*	
A	−452	5′ GGAAGGGCTAATTCACTCACAG 3′	−431
B	+123	3′ ACTGAGACCATTGATCTCTAG 5′	+145
C	−395	5′ CACACAAGGCTACTTCCCTGA 3′	−375
D	+44	5′ GATTGATCCCTTGGGTGACG 3′	+63
E	−9	5′ GCCTGTACTGGGTCTCTCTGG 5′	+11

Materials and methods

The right halves of brains from HIV-infected adult patients from New York and Minneapolis were collected at autopsy, 6–48 h after death, and fresh frozen. Approximately 0.5 cm × 0.5 cm × 0.5 cm pieces of right frontal gray and subcortical white matter were chipped from each brain using a sterile, disposable scalpel, taking care to minimize exposure to blood components. The pieces were chopped with the scalpel, pulverized in a disposable 1.5 ml plastic tube with a disposable pestle, and treated with proteinase K (50 μg/ml) in 1% SDS, 50 mM Tris (pH 8.0), 100 mM EDTA, and 100 mM NaCl overnight with shaking at 55°C. The mixture was treated with RNAse A, 1 μg/ml, at 37°C for 30 min, extracted with phenol, phenol/chloroform and chloroform, precipitated with 100% ethanol and 0.3 M NaAcetate, washed with 70% ethanol, dried, resuspended in TE 8.0 and quantitated. One μg of genomic DNA was used as a template for polymerase chain rection (PCR) using GAPDH primers, to determine if the DNA was intact.

Portions of the HIV-1 LTR were amplified by PCR using either primers A and B, C and B, or a nested set of primers, A and B, followed by C and D (Table 1). Those portions of the LTR used as primers were identical in the IIIB, JR-CSF and JR-FL clones. One or two rounds of PCRs were used to derive all of the sequences from each brain section. Amplification consisted of 35 cycles of 60 s at 94°C, 45 s at 55–59°C and 60 s at 72°C, followed by one cycle for 7 min at 72°C. The 40 μL reaction mixture contained 1 μg of genomic DNA, 80 μM of each primer and 20 mM each of dATP, dCTP, dGTP and dTTP in 10 m Tris, 1.5 mM $MgCl_2$ and 50 mM KCl. PCR products were ligated into a TA cloning vector (Invitrogen) overnight, *E. coli* INVαF′ cells were transformed with the ligation mix, and miniprep DNA was

prepared (BIO-RAD). After a diagnostic restriction digest, the DNA was sequenced manually with the chain termination technique using Sequenase T7 DNA polymerase (Amersham), primers T7 and SP6, and an internal primer, E (Table 1), from the LTR. An overall error rate of one base per $1-5\times10^5$ bases was determined by amplifying and sequencing analogous sequences of known plasmid DNA.

The sequences were read manually and entered into a Compaq Prolinea 4/50 computer for phylogenetic analysis by the Lasergene software (DNASTAR). For purposes of this discussion, the term quasispecies is defined as a population of closely related genomes, apparently derived from a common progenitor (Delassus *et al*, 1991; Goodenow *et al*, 1989). Clones were named by the brain from which they were derived, a designation for white or gray matter (w or g, respectively), and a sequential clone number from that brain, e.g. 2135W1, 2135W2, etc. The 't' after each sequence refers to the fact that these sequences are truncated, and do not include the primers used to amplify them.

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