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Development of co-selected single nucleotide polymorphisms in the viral promoter precedes the onset of human immunodeficiency virus type 1-associated neurocognitive impairment

Luna Li,

Department of Microbiology and Immunology, Drexel University College of Medicine, 245 N. 15th Street, MS #1013A, Philadelphia, PA 19102, USA. Center for Molecular Virology and Translational Neuroscience, Institute for Molecular Medicine and Infectious Disease, Drexel University College of Medicine, Philadelphia, PA, USA

Benjamas Aiamkitsumrit,

Department of Microbiology and Immunology, Drexel University College of Medicine, 245 N. 15th Street, MS #1013A, Philadelphia, PA 19102, USA. Center for Molecular Virology and Translational Neuroscience, Institute for Molecular Medicine and Infectious Disease, Drexel University College of Medicine, Philadelphia, PA, USA

Vanessa Pirrone,

Department of Microbiology and Immunology, Drexel University College of Medicine, 245 N. 15th Street, MS #1013A, Philadelphia, PA 19102, USA. Center for Molecular Virology and Translational Neuroscience, Institute for Molecular Medicine and Infectious Disease, Drexel University College of Medicine, Philadelphia, PA, USA

Michael R. Nonnemacher,

Department of Microbiology and Immunology, Drexel University College of Medicine, 245 N. 15th Street, MS #1013A, Philadelphia, PA 19102, USA. Center for Molecular Virology and Translational Neuroscience, Institute for Molecular Medicine and Infectious Disease, Drexel University College of Medicine, Philadelphia, PA, USA

Adam Wojno,

Department of Microbiology and Immunology, Drexel University College of Medicine, 245 N. 15th Street, MS #1013A, Philadelphia, PA 19102, USA. Center for Molecular Virology and Translational Neuroscience, Institute for Molecular Medicine and Infectious Disease, Drexel University College of Medicine, Philadelphia, PA, USA

Shendra Passic,

Department of Microbiology and Immunology, Drexel University College of Medicine, 245 N. 15th Street, MS #1013A, Philadelphia, PA 19102, USA. Center for Molecular Virology and Translational Neuroscience, Institute for Molecular Medicine and Infectious Disease, Drexel University College of Medicine, Philadelphia, PA, USA

Katherine Flaig,

Department of Microbiology and Immunology, Drexel University College of Medicine, 245 N. 15th Street, MS #1013A, Philadelphia, PA 19102, USA. Center for Molecular Virology and

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Correspondence to: Brian Wigdahl, bwigdahl@drexelmed.edu.

Luna Li and Benjamas Aiamkitsumrit have contributed equally to this manuscript.

Translational Neuroscience, Institute for Molecular Medicine and Infectious Disease, Drexel University College of Medicine, Philadelphia, PA, USA

Evelyn Kilareski,

Department of Microbiology and Immunology, Drexel University College of Medicine, 245 N. 15th Street, MS #1013A, Philadelphia, PA 19102, USA. Center for Molecular Virology and Translational Neuroscience, Institute for Molecular Medicine and Infectious Disease, Drexel University College of Medicine, Philadelphia, PA, USA

Brandon Blakey,

Department of Microbiology and Immunology, Drexel University College of Medicine, 245 N. 15th Street, MS #1013A, Philadelphia, PA 19102, USA. Center for Molecular Virology and Translational Neuroscience, Institute for Molecular Medicine and Infectious Disease, Drexel University College of Medicine, Philadelphia, PA, USA

Jade Ku,

Department of Microbiology and Immunology, Drexel University College of Medicine, 245 N. 15th Street, MS #1013A, Philadelphia, PA 19102, USA. Center for Molecular Virology and Translational Neuroscience, Institute for Molecular Medicine and Infectious Disease, Drexel University College of Medicine, Philadelphia, PA, USA

Nirzari Parikh,

Department of Microbiology and Immunology, Drexel University College of Medicine, 245 N. 15th Street, MS #1013A, Philadelphia, PA 19102, USA. Center for Molecular Virology and Translational Neuroscience, Institute for Molecular Medicine and Infectious Disease, Drexel University College of Medicine, Philadelphia, PA, USA

Rushabh Shah,

Department of Microbiology and Immunology, Drexel University College of Medicine, 245 N. 15th Street, MS #1013A, Philadelphia, PA 19102, USA. Center for Molecular Virology and Translational Neuroscience, Institute for Molecular Medicine and Infectious Disease, Drexel University College of Medicine, Philadelphia, PA, USA

Julio Martin-Garcia,

Department of Microbiology and Immunology, Drexel University College of Medicine, 245 N. 15th Street, MS #1013A, Philadelphia, PA 19102, USA. Center for Molecular Virology and Translational Neuroscience, Institute for Molecular Medicine and Infectious Disease, Drexel University College of Medicine, Philadelphia, PA, USA

Brian Moldover,

B-Tech Consulting, Ltd., Philadelphia, PA, USA

Laila Servance,

Division of Infectious Disease and HIV Medicine, Department of Medicine, Drexel University College of Medicine, Philadelphia, PA, USA

David Downie,

Division of Infectious Disease and HIV Medicine, Department of Medicine, Drexel University College of Medicine, Philadelphia, PA, USA

Sharon Lewis,

Division of Infectious Disease and HIV Medicine, Department of Medicine, Drexel University College of Medicine, Philadelphia, PA, USA

Jeffrey M. Jacobson,

Department of Microbiology and Immunology, Drexel University College of Medicine, 245 N. 15th Street, MS #1013A, Philadelphia, PA 19102, USA. Center for Molecular Virology and Translational Neuroscience, Institute for Molecular Medicine and Infectious Disease, Drexel

University College of Medicine, Philadelphia, PA, USA. Center for Clinical and Translational Medicine, Institute for Molecular Medicine and Infectious Disease, Drexel University College of Medicine, Philadelphia, PA, USA. Division of Infectious Disease and HIV Medicine, Department of Medicine, Drexel University College of Medicine, Philadelphia, PA, USA

Dennis Kolson, and

Department of Neurology, University of Pennsylvania School of Medicine, Philadelphia, PA, USA

Brian Wigdahl

Department of Microbiology and Immunology, Drexel University College of Medicine, 245 N. 15th Street, MS #1013A, Philadelphia, PA 19102, USA. Center for Molecular Virology and Translational Neuroscience, Institute for Molecular Medicine and Infectious Disease, Drexel University College of Medicine, Philadelphia, PA, USA. Center for Clinical and Translational Medicine, Institute for Molecular Medicine and Infectious Disease, Drexel University College of Medicine, Philadelphia, PA, USA

Brian Wigdahl: bwigdahl@drexelmed.edu

Abstract

The long terminal repeat (LTR) regulates gene expression of HIV-1 by interacting with multiple host and viral factors. Cross-sectional studies in the pre-HAART era demonstrated that single nucleotide polymorphisms (SNPs) in peripheral blood-derived LTRs (a C-to-T change at position 3 of C/EBP site I (3T) and at position 5 of Sp site III (5T)) increased in frequency as disease severity increased. Additionally, the 3T variant correlated with HIV-1-associated dementia. LTR sequences derived by longitudinal sampling of peripheral blood from a single patient in the DrexelMed HIV/AIDS Genetic Analysis Cohort resulted in the detection of the 3T and 5T co-selected SNPs before the onset of neurologic impairment, demonstrating that these SNPs may be useful in predicting HIV-associated neurological complications. The relative fitness of the LTRs containing the 3T and/or 5T co-selected SNPs as they evolve in their native patient-derived LTR backbone structure demonstrated a spectrum of basal and Tat-mediated transcriptional activities using the IIIB-derived Tat and colinear Tat derived from the same molecular clone containing the 3T/5T LTR SNP. In silico predictions utilizing colinear envelope sequence suggested that the patient's virus evolved from an X4 to an R5 swarm prior to the development of neurological complications and more advanced HIV disease. These results suggest that the HIV-1 genomic swarm may evolve during the course of disease in response to selective pressures that lead to changes in prevalence of specific polymorphisms in the LTR, *env*, and/or *tat* that could predict the onset of neurological disease and result in alterations in viral function.

Keywords

HIV-1; SNP; HAND; LTR; Env

Introduction

The progression of human immunodeficiency virus type 1 (HIV-1)-associated pathogenesis and disease depends on the ability of the virus to infect and replicate within susceptible cells of the immune and central nervous systems (CNS). HIV-1 invades host cells by binding to the cellular receptor CD4 and one of the chemokine co-receptors, CXCR4 or CCR5, to enter a number of different target cells including T cells or cells of the monocyte-macrophage lineage (Fields et al. 2001). HIV-1 infection of activated CD4⁺ T lymphocytes is highly productive with periodic bursts of replication in regional lymph nodes that ultimately lead to a slow but steady decline of CD4⁺ T cells over the course of disease, which ultimately results in the onset of acquired immunodeficiency syndrome (AIDS) (Pantaleo et al. 1993).

HIV-1 infection of cells of the monocyte–macrophage lineage represents a chronic infection without destruction of the host cell and with a less productive replication cycle, leading to the synthesis and release of lower quantities of infectious virus. HIV-1 infection of this cell population during treatment with highly active antiretroviral therapy (HAART) may be of particular importance because cells of the monocytic lineage may represent one potential reservoir for continued viral replication based on the chronic nature of this virus–cell interaction (Chun et al. 1997); this observation is particularly true for monocytic cells after tissue penetration. HIV-1-infected monocytes have also been shown to be involved in transporting HIV-1 across the blood–brain barrier into the CNS where perivascular macrophages and microglial cells serve as the major populations of cells infected with HIV-1 within the brain. HIV-1-infected cells of the monocyte–macrophage lineage including brain microglial cells serve as the major source of infectious virus in the brain, and virus produced by these cells is likely responsible for infection of the astrocyte compartment within the brain.

Because viral replication in cells of the monocyte–macrophage lineage, including microglial cells, likely plays an important role in the genesis of neurological dysfunction in the absence or presence of antiretroviral therapy, we have been interested in defining the cellular and viral factors involved in regulating viral transcription in a number of cellular phenotypes targeted by HIV-1 during the course of infection. HIV-1 transcription is mediated by host cell transcription factors and viral proteins that bind to *cis*-regulating elements in the viral promoter, designated the long terminal repeat (LTR) (Fields et al. 2007). LTR basal and stimulated transcription is driven primarily through cellular transcription factors such as Sp1 and nuclear factor kappa light-chain enhancer of activated B cells (NF- κ B), which have been shown to produce predominantly short RNAs as a result of the hypophosphorylated state of RNA pol II. However, longer transcripts are produced at low abundance that encode for an increasing quantity of early viral regulatory proteins, including the transactivator protein (Tat), that eventually feedback to enhance the next stage of viral transcription, referred to as Tat-mediated transactivation, which enhances production of the long transcripts encoding more Tat and increased synthesis of structural proteins.

In addition to the high levels of HIV-1 gene expression observed in activated CD4⁺ T cells, HIV-1 replication in cells of the monocyte–macrophage lineage has been shown to be more chronic with lower levels of gene expression. The viral envelope proteins gp120 and gp41, along with differential expression and utilization of the co-receptors CXCR4 and CCR5, dictate the efficacy with respect to viral entry into cells with different phenotypes and leads to cell type-specific replication as well as the overall level of gene expression in a given cellular compartment. However, subsequent to viral entry, the HIV-1 gene expression program may be guided by specialized *cis*-acting regulatory elements within the viral LTR and/or differential utilization of cellular transcription factors with cell type-specific, activation/differentiation-specific patterns of expression and/or covalent modification of DNA. In addition, cell type-specific differences in patterns of HIV-1 proviral DNA integration and epigenetic controls may also be responsible for guiding differential gene expression in cells of differing phenotypes. With regard to HIV-1 replication in cells of the monocyte–macrophage lineage, the CCAAT/enhancer binding protein (C/EBP) binding sites, mapping upstream of the NF- κ B core/enhancer region, have been shown to be indispensable for HIV-1 replication in monocyte–macrophages but dispensable for replication in T cells (Henderson and Calame 1997; Henderson et al. 1995, 1996). In addition, recruitment of Sp transcription factors to Sp site III could be of greater importance to the function of the viral promoter in T cells than in cells of the monocyte–macrophage lineage (McAllister et al. 2000). These studies demonstrated that *cis*-acting elements within the LTR may exhibit distinct patterns of utilization in T cells and monocytic cells, indicating

that cell-type specific transcription factors could be used by properly adapted viral promoter sequences and may have impact on the overall fitness of the LTR and general viral fitness.

HIV-1 genotypic variation and the potential for altered phenotype occur as important variables of viral replication during the course of disease (Zhu et al. 1993). HIV-1 quasiespecies contain sequence changes throughout the viral genome, including the LTR (Fields et al. 2007; Gao et al. 1996). These sequence changes may result in altered LTR fitness and overall viral gene expression. Both blood-derived HIV-1 and brain-derived viruses show molecular heterogeneity between patients and subtypes (Ross et al. 2001). Several HIV-1 genes including *tat*, *env*, *vpr*, and *nef*, as well as the viral LTR, have been shown to be involved in the pathogenesis of HIV-related neurological disease (van Marle and Power 2005). Phylogenetic and sequence comparisons of HIV-1 genes derived from brain and other tissues indicate that brain-specific HIV-1 quasiespecies exist (Hughes et al. 1997; Peters et al. 2007). In studies performed with clinical samples derived from HIV-1-infected patients in the pre-HAART era, single nucleotide changes were observed within the NF- κ B-proximal C/EBP and Sp transcription factor binding sites at greater frequency in LTRs isolated from HIV-1-infected patients with more severe disease than those from individuals with less severe HIV disease. These LTRs contained specific single nucleotide polymorphisms (SNPs) in C/EBP site I (a C-to-T change at position 3 of a consensus B C/EBP binding site I: 3T) that were more frequent in tissues derived from individuals who had died with HIV-associated dementia (HAD) than those who died without HAD (McAllister et al. 2000; Ross et al. 2001). It has been reported that specific LTR SNPs within C/EBP site I (3T) and Sp site III (a C-to-T change at position 5 of a consensus B Sp binding site: 5T) increase in prevalence coordinately over the course of HIV-1 infection and correlate with disease severity (Hogan et al. 2003; Nonnemacher et al. 2004).

Herein, we describe the enrollment of HIV-1-infected patients into the DREXELMED HIV/AIDS Genetic Analysis Cohort. Within this cohort, a number of patients carrying the 3T and/or 5T SNPs were identified. Patient 107 of this cohort was studied in detail to demonstrate the potential power of these two SNPs with regard to prediction of increased disease progression and neurological impairment. The studies performed explore the relative fitness of the HIV-1 LTRs containing the 5T knockout configuration of consensus B Sp site III alone or the 3T/5T co-selected knockout configurations of consensus B C/EBP site I and Sp site III as they evolve in their native patient-derived LTR backbone structure. The evolution of the viral proteins Tat and Env is also examined during the course of disease in this patient.

Results

The DREXELMED HIV/AIDS genetic analysis cohort contains patients with the co-selected 3T/5T viral LTR genetic signature

Studies in the pre-HAART era demonstrated position 3 of C/EBP site I and position 5 of Sp site III to be more variant than the other nucleotide positions within that given site and correlated to HIV-1 disease progression (Hogan et al. 2003; Nonnemacher et al. 2004; Ross et al. 2001). They also demonstrated that the 3T C/EBP site I correlated to patients diagnosed with HAD (Hogan et al. 2003). We therefore attempted to determine whether HAART-experienced patients harbor the 3T C/EBP site I and 5T Sp site III SNPs. To address this issue to date, we have enrolled 434 patients being cared for in the Drexel University College of Medicine HIV/AIDS clinic, located in Philadelphia, Pennsylvania, USA, into the DREXELMED HIV/AIDS Genetic Analysis Cohort to assess the role that genetic variation within the HIV-1 sequence plays in disease progression and potentially in the development of neurological complications. We collected blood from these 434 patients

for drug testing and viral genetic assessment and determined clinical parameters for each patient at each visit (total of 869 visits) as described in “Materials and methods.”

Peripheral blood mononuclear cells (PBMCs) were isolated from the blood samples collected from each patient at each visit (869 visits total), and a fraction of these cells was used for genomic DNA isolation, from which integrated proviral DNA was amplified as described below, to ascertain the sequence of the LTR. The sequences obtained from each patient were indicative of the predominant sequence seen in the viral quasispecies because these sequences were taken directly from PCR products and were not cloned. At the time of this manuscript, 701 patient visits had been successfully sequenced and analyzed manually. Of these 701 LTR sequences, 633 were analyzed using Phred (Brockman et al. 2008), trimmed using a quality score of 20 as a threshold (P error= 0.01%) for sequences having a length >100 nucleotides, and SNPs identified using the Mutation Surveyor package as described in the “Materials and methods.” Cloning of these PCR products demonstrates that these are in fact predominant species, with other less abundant species also present (data not shown). The sequences for each patient from each visit obtained were aligned to the Los Alamos-derived consensus subtype B (ConB) sequence from January 2002. The LTR sequence was further assessed for genetic variation within 12 main transcription factor binding sites of interest: C/EBP site II, upstream stimulating factor (USF) binding site, E-twenty six-1 (Ets-1) binding site, lymphoid enhancer binding factor-1 (Lef-1) binding site, activating transcription factor/cyclic AMP response element-binding (ATF/CREB) binding site, C/EBP site I, NF- κ B site II, NF- κ B site I, stimulatory protein (Sp) site III, Sp site II, Sp site I, and octamer binding protein-I (Oct-I) binding site.

The 3T C/EBP site I variant that was previously identified is present in the DREXELMED HIV/AIDS Genetic Analysis Cohort, albeit at a low frequency (3.48% of LTRs, 22 of 633 visits). Of the 22 visits analyzed, there are two with undetermined nadir CD4 counts. Of the 20 visits with reported nadir CD4 T cell counts, the average nadir CD4 T cell count is 264 cells/ μ L. Of these 20 visits, 15 were on continuous HAART (cH) with an average nadir CD4 T cell count of 228 cells/ μ L, 2 were on discontinuous HAART (dH) with an average nadir CD4 T cell count of 116 cells/ μ L, and 3 were naïve to HAART (nH) with an average nadir CD4 T cell count of 540 cells/ μ L. Of the 17 visits of patients with cH or dH, their average nadir CD4 T cell count is 215 cells/ μ L. Given that patients are typically only given HAART therapy once their counts go below 250 cells/ μ L, this observation is consistent with earlier observations that this variant becomes more prevalent when the severity of HIV disease increases. The 5T Sp site III variant also occurs in the DREXELMED cohort at a frequency of 115 sequences from 633 total sequences or 18.2% of LTRs. In comparison to a number of published reports (Burdo et al. 2004; Hogan et al. 2003; McAllister et al. 2000; Nonnemacher et al. 2004; Ross et al. 2001), in the pre-HAART era, these frequencies would seem to indicate that as a population of HIV-1-infected patients, the DREXELMED HIV/AIDS Genetic Analysis Cohort is relatively healthy and in fact has an average most recent viral load of 25,266 RNA copies per milliliter and most recent CD4 count of 466 cell/ μ L.

Patient 107 contains the 3T C/EBP site I and 5T Sp site III

Of the 22 visits that were identified to have a 3T at C/EBP site I, 19 had been given the mini-bedside neurocognitive assessment. Of these 19, eight had a neurocognitive score below 10; this represents seven patients. Of these seven patients, patient 107 and patient 110 R01 had the 3T/5T combination SNP at the time neurocognitive impairment was assessed. However, patients 213 R00 (initial visit) and patient 247 R00 (initial visit) exhibited the 3T/5T marker but did not show neurocognitive impairment at this point in time. Unfortunately, patient 213 has not been seen for their second visit and at this time is therefore considered lost to follow-up (although we continue to try to obtain additional samples from this patient). Patient 247 at the time of their second visit had a drop in their neurocognitive score from

11.5 to 9.5, which would again have been predicted by the 3T/5T marker; however, a LTR sequence from the R01 visit has not yet been obtained. From this assessment, patient 107 became of particular interest based on the sequence alignment (Fig. 1) and on genetic variations within the binding sites of interest from longitudinal samples (Table 1) showing the 3T and 5T variations of particular interest in C/EBP site I and Sp site III, respectively. During the first visit (R00), patient 107 displayed a 3T/ConB genotype at C/EBP site I and Sp site III, respectively. The sequence changed to 3T/5T at the second visit (R01), remained 3T/5T at visit 3 (R02), and converted to a 3T6G/ConB at visit 4 (R03), then subsequently reverted to 3T/5T at visit 5 (R04).

The 3T C/EBP site I and 5T Sp site III genotypes in the HIV-1 LTR precede HIV-1 disease progression

Because of this genetic signature, patient 107 was analyzed to determine if the presence of the 3T/5T genotype was predictive or indicative of increased disease progression. Patient 107, diagnosed as HIV-1-positive in 2004, was first enrolled in the DREXELMED HIV/AIDS Genetic Analysis Cohort in 2007, with two subsequent visits in 2008 and one visit in 2009. Throughout these four visits, patient 107 has consistently remained hepatitis B- and C-negative while consistently testing negative for drugs of abuse (Table 2). For visits R00 through R02, patient 107 was actively on HAART therapy; however, at the R03 visit, HAART was discontinued due to toxicity. During HAART use, the CD4⁺ T cell count at each visit remained high, ranging from 565 cells/ μ L at R00 to 720 cells/ μ L at R02. At R03, when the patient was no longer taking HAART, the CD4 count dropped to 95 cells/ μ L. This drop in CD4 count reset the nadir for this patient, which was previously 162 cells/ μ L. During each of these visits, there was no discernable change in viral load, with a range in current viral load from a low of 64 RNA copies per milliliter at R02 to a high of 154 RNA copies per milliliter at R03 (Table 2). Interestingly, these data suggest that the presence of the 3T/5T LTR genetic signature may occur prior to the onset of disease progression.

The co-selected 3T C/EBP site I and 5T Sp site III genotype in the HIV-1 LTR precedes the development of HIV-1 neurological disease

Patient 107 was assessed at each visit for potential neurological impairment. At the R00 visit, the patient was assessed using the Memorial Sloan-Kettering criteria for HAD (Price and Brew 1988). At this visit, patient 107 was classified with a normal neurological score while harboring a 3T C/EBP site I variant. At the R01 visit, using the modified IHD test (Fig. 2a), the patient scored 12, which is classified as a normal neurological score, while harboring the 3T/5T LTR genotype. At the R02 visit, the patient began to demonstrate signs of neurological impairment, scoring 8/12 on the modified IHD test. In addition, the patient also went from a perfect 2/2 constructional score at R01 to a 0/2 constructional score, with a cube that did not demonstrate any three-dimensional quality (Fig. 2b). The R03 visit was similar to the R02 visit, with an impaired neurological score of 9.5 and a 0/2 constructional score, with a cube demonstrating no three-dimensional quality. The decline in neurologic score correlated with the presence of the 3T/5T LTR genotype as observed in the pre-HAART era, suggesting that the presence of these co-selected variants, as the predominant species in the viral swarm, may occur prior to the onset of neurological decline.

HIV-1 LTRs containing the 3T/5T genotype are functional and responsive to Tat

Because the 5T sequence configuration of Sp site III (Nonnemacher et al. 2004) and the 3T sequence configuration of C/EBP site I (Ross et al. 2001) have been shown to be knockout binding sites for their cognate factors, we proceeded to determine whether the patient-derived LTRs containing either or both of the configurations could have basal transcription activity similar to that of the laboratory strain HIV-1_{LAI} LTR or would they be defective based on the knockout phenotype of the binding sites. In this regard, the patient-derived

HIV-1 LTR-driven luciferase constructs were transiently transfected into the CD4⁺ Jurkat T cell line in parallel with luciferase reporter constructs driven by the HIV-1 LAI LTR and the LAI LTR containing the 5T variation at Sp site III. For studies presented here, in addition to patient 107, patients 49, 50, and 119 were selected for comparison because they contained a 5T at Sp site III of the HIV-1 LTR, and patient 20 was selected as a control because the LTRs contained a conB Sp site III. The relative basal activity of each LTR clone is shown as the fold over LAI LTR basal activity, which ranges from 0.4 to 2.0 (Fig. 3a). The LAI 5T LTR and patient-derived LTR clones including 20-1, 49-1, 49-2, 50-4, 50-9, 107-15, 107-52, and 107-65 did not show different basal transcription activity compared to LAI LTR. However, patient-derived LTR clones such as 20-8, 49-17, 107-5, 107-13, 119-7, and 119-14 exhibited a statistically significant difference ($P<0.01$) in basal transcription activity compared with LAI LTR. However, the LTRs containing the 5T Sp site III exhibited basal transcription activities similar to those of LTRs containing a conB configuration of Sp site III. For example, both the clones 119-7 LTR and 119-14 LTR, which contain the conB configuration of Sp III and 5T Sp III, respectively, exhibited 0.4- or 0.6-fold LTR activation as compared to the LAI basal activity in Jurkat T cells. Overall, these results indicated that the panel of LTRs containing the 5T configuration of Sp site III derived from a number of HIV-1-infected patients was similar to that of the LAI LTR, or the patient-derived conB Sp site III LTRs, with respect to the capability of driving basal transcription in T cells.

Similar transfection experiments were also performed in the U-937 monocytic cell line. The fold over LAI LTR basal activity for each LTR ranges from 0.4 to 2.1. No significant difference was observed between most patient-derived LTRs and the LAI LTR or LAI LTR containing the 5T configuration of Sp site III, except clones 107-15, 107-52, and 107-65 (Fig. 3b). As demonstrated with the panel of HIV-1-infected, patient-derived LTRs in Jurkat T cells, the 5T Sp site III containing LTRs did not exhibit a different basal transcription profile when compared to the panel of conB Sp site III containing LTRs. The results obtained from the transient transfection studies performed in the T cell and monocytic cell lines demonstrated that 5T Sp site III and/or 3T (C/EBP I)/5T (Sp site III) co-selected LTR configurations do not affect the basal transcriptional activity of these patient-derived LTRs. However, selected LTR clones such as 107-5, 107-15, and 107-65 showed different patterns of basal transcriptional activities in the two cell lines, suggesting that cellular phenotype may alter functional properties of 5T- and/or 3T/5T-containing HIV-1 LTRs.

The next question that was addressed was to determine whether the HIV-1-infected patient-derived LTRs could be transactivated by the transactivator protein Tat (derived from the widely used laboratory strain HIV-1_{IIIB}; Tat86 is a naturally occurring deletion of 15 amino acids from carboxy terminus of exon II). To this end, co-transfection studies were performed with each LTR construct and the HIV-1_{IIIB} Tat86 expression vector in Jurkat T cells (Fig. 3a). These studies indicated that the fold over LAI LTR basal activity ranged from ~12 with the 119-7 LTR to ~61 with the 49-17 LTR. However, when Tat-mediated transactivation of each LTR was compared to the basal activity of the same LTR rather than to the activity obtained with the LAI LTR, a majority of the patient-derived HIV-1 LTRs exhibited approximately a 30- to 46-fold increase in transcription in response to Tat (Fig. 3a). Co-transfections of each LTR construct with the IIIB Tat86 expression vector were also performed in U-937 monocytic cells (Fig. 3b). These studies yielded fold over LAI LTR basal activity values ranging from ~15 with the 20-8 LTR to ~170 with the 107-15 LTR. When Tat-mediated transactivation of each LTR was compared with the basal activity of the same LTR rather than with the activity obtained with the LAI LTR, the patient-derived HIV-1 LTRs containing the 5T Sp III exhibited approximately a 20-fold increase with the 20-8 LTR to 200-fold with the 107-65 LTR with respect to IIIB Tat transactivation (Fig. 3b). The results presented demonstrate that the patient-derived HIV-1 LTRs containing the 5T configuration of Sp III exhibited Tat-mediated transcription activation levels similar to those

of the LTRs containing the conB configuration of Sp site III in both Jurkat cells and U-937 cells. In addition, 3T (C/EBP site I)/5T (Sp site III)-containing LTR clones (clones 107-5, 107-15, and 107-65) demonstrated different patterns of Tat transactivation in the two cell lines, again suggesting that cellular phenotype may alter functional properties of 5T- and/or 3T/5T-containing LTRs.

Patient 107 Tat transactivates the corresponding HIV-1-infected patient-derived colinear LTR better than a non-colinear Tat protein in Jurkat T cells

To study the functional properties of the patient 107-derived Tat proteins, full-length *tat* genes from R00 and R01 were cloned into the expression vector pcDNA3.1(+)/Hygro. These clones were selected for analysis based on the representative nature of their sequence as compared to those of other LTR clone sequences derived from each visit. The general transactivation capability of the HIV-1-infected, patient-derived Tat proteins with respect to the HIV-1 LAI LTR was determined. To this end, co-transfection studies were performed with each HIV-1-infected, patient-derived Tat expression vector and the HIV-1 LAI LTR construct in Jurkat T cells (data not shown). All five HIV-1-infected, patient-derived full-length Tat101 clones dramatically induced LTR transcription more than 40-fold over LAI LTR basal activity. HIV-1 Tat clones 19-c1 Tat101, 107-R01 Tat101, and 131-c1 Tat101 exhibited a similar level of transactivation as HIV-1 IIIB Tat101, whereas 107-R00 Tat101 and 119-c2 Tat101 exhibited a lower level of transactivation than that obtained with HIV-1 IIIB Tat101.

To address this observation in more detail, “cross” co-transfection studies were performed with clone 107-R00 Tat101 and 107-R01 Tat101 expression vectors and the corresponding colinear 107-c1 LTR and 107-R01 LTR constructs in Jurkat T cells (Fig. 4). Both 107-R00 Tat101 and 107-R01 Tat 101 exhibited a higher level of trans-activation with the 107-R00 LTR than with the HIV-1 IIIB Tat101 ($P=0.042$ with 107-R00 Tat101 and $P=0.045$ with 107-R01 Tat 101). In addition, both 107-R00 Tat101 and 107-R01 Tat101 exhibited a significantly higher level of transactivation with respect to the 107-R01 LTR compared with the level achieved with HIV-1 IIIB Tat101 ($P<0.01$). Interestingly, 107-R01 Tat101 could transactivate the colinear LTR at a higher level than 107-R00 Tat101 ($P=0.015$), whereas 107-R00 Tat101 did not show much difference from 107-R01 Tat101 with respect to 107-R00 LTR transcription. These results suggest that the Tat protein and LTR may be undergoing a process of adaptation during at least a part of the disease course that favors increased transactivation.

Patient 107-derived HIV-1 Tat clones have non-consensus variations compared with IIIB or the consensus B sequence of Tat

To understand what amino acid changes may have occurred to cause the increase in Tat transactivation from R01, sequence alignments of patient 107-derived Tat proteins were compared to the laboratory strain IIIB and conB Tat using the CLUSTALW algorithm. Each of the HIV-1 Tat clones examined displayed a number of amino acid alterations. Compared to the HIV-1 IIIB or conB Tat sequences, clones 107-R00 Tat and 107-R01 Tat both contain several non-consensus variations including N24P (asparagine to proline change at residue 24), I39T (isoleucine to tyrosine change at residue 39), V67G (valine to glycine change at residue 67), S68Y (serine to tyrosine change at residue 68), P/S77H (proline or serine to histidine change at residue 77), and F/V100K (phenylalanine or valine to lysine change at residue 100; Fig. 5). Clone 107-R00 Tat and 107-R01 Tat also contained a few conservative sequence alterations such as H65D (histidine to aspartic acid change at residue 65), S70P and S75P (serine to proline change at residues 70 and 75), and D98G (aspartic acid to glycine change at residue 98). In addition to these sequence alterations, clone 107-R00 Tat contained a number of more conservative amino acid alterations with A/T74S (alanine or

threonine to serine change at residue 74), whereas clone 107-R01 Tat was shown to have two additional conservative changes: P6S (proline to serine change at residue 6) and A/T74G (alanine or threonine to glycine change at residue 74).

Patient 107 envelope changes from an X4-utilizing to an R5-utilizing co-receptor virus prior to neurological complications and increased disease progression

During the course of HIV-1 infection, it is thought that the co-receptor usage of the virus will start as an R5-utilizing virus, adapt to an X4- or X4/R5-utilizing virus, and then finally go back to an R5-utilizing virus (Ho et al. 2007; Kitchen et al. 2004). This pattern is thought to correlate with what co-receptors are available on cells specifically found at each stage of disease. Given that the LTR acquired changes that were predictive of neurological impairment and that Tat and the LTR seemed to adapt in a way that increased transcriptional ability in the R01 visit, studies proceeded to determine if the envelope was also adapting during this time. To address this question, four different *in silico* prediction methods [sequence observation analysis (11/25 rule), ProtParam, Geno2pheno, and PSSM] were used to determine the co-receptor usage of the viral quasispecies at each visit. These methods were performed on 1 clone from the R00, 11 clones from the R01, 3 clones from the R02, and 10 clones from the R03 visits for patient 107 (Table 3). All four prediction methods use slightly different parameters to determine co-receptor usage; however, for each sequence examined here, all the prediction methods were in agreement. At the first visit (R00), the envelope was determined to be X4-utilizing. At each subsequent visit, the envelope had changed to an R5-utilizing phenotype. The only exception was one clone from the R02 visit predicted to be an X4-utilizing virus, whereas the rest were predicted to be R5-utilizing. These results suggested that the envelope may be adapting from replicating predominantly in T cells (primarily X4) to replicating predominantly in cells of monocyte-macrophage origin (primarily R5), the cell type responsible for carrying the virus to the brain and contributing to neuropathogenesis.

Discussion

We previously used clinical and HIV genomic sequence information derived from integrated HIV-1 provirus contained within the PBMC compartment to begin to identify and characterize HIV-1 LTR sequence signatures that may correlate with the severity of HIV disease or be predictive of those HIV-1-infected patients from the pre-HAART era who may be more prone to the development of HIV-1-associated neurological disease (Burdo et al. 2004; Hogan et al. 2003; McAllister et al. 2000; Nonnemacher et al. 2004; Ross et al. 2001). These early studies led to the hypothesis that during the course of HIV-1 disease, the LTR 5T Sp site III variant may be found more prevalently in earlier disease, with the 3TC/EBP site I variant arising later in the progression of the disease in the peripheral blood compartment. Because of HAART, the immune response, and the chronic nature of HIV disease, it has been proposed that genetic variations may accumulate over time and become more or less prevalent within the viral quasispecies present in the peripheral blood.

When one examines patient 107 closely, it is evident that the 3T and/or 5T SNPs could be identified prior to the onset of disease progression, as observed with the decrease in CD4⁺ T cell count in R03 (compare Tables 1 and 2). During this time frame, the envelope co-receptor utilization switched from X4-utilizing (R00 visit) to R5-utilizing (R01, Table 3). This result suggested that the virus is under more pressure to replicate in monocytic cells than in T cells. This observation may be due to the fact that HAART suppressed viral replication during this time, particularly in T cells. In fact, it is important to note that the changes observed in the LTR and *env* gene are occurring while the patient is on HAART therapy (Table 2). Finally, both the presence of the 3T and 5T SNPs and the switch to an R5-utilizing co-receptor phenotype occurred prior to the decrease in neurocognitive

performance in the mini-neurological examination. However, two limitations exist with these analyses. First, only one R00 clone was examined to date, leaving the possibility that the R00 visit was a mixed R5X4 population. If this is true, then what is being observed potentially changes in relative abundance of each type of virus within the quasispecies of the patient. Therefore, more clones and additional time points will need to be analyzed. Second, these are *in silico* prediction models. While each of these models has been shown to predict co-receptor usage, these predictions need to be analyzed using *in vitro* functional assays.

Many studies have demonstrated the impact of cellular proteins on viral entry processes, early viral replicative events, integration, and viral gene expression, including the control of viral transcription and translation, protein modification, intracellular localization, and viral assembly and egress. Some of these studies demonstrated the importance of the three Sp binding sites in LTR-directed basal transcription and gene expression (Jones et al. 1986; Pereira et al. 2000) and replication (Ross et al. 1991). Specifically, Sp site III sequence variants have been identified to affect both viral replication and LTR function in Jurkat T cells and cells of monocyte–macrophage lineage (McAllister et al. 2000). Likewise, with regard to HIV-1 replication in cells of the monocyte–macrophage lineage, C/EBP binding sites have been shown to be indispensable for HIV-1 monocytic replication but dispensable for replication in T cells (Henderson and Calame 1997; Henderson et al. 1995, 1996). Previous studies also indicated a strong correlation between HIV-1 LTR-directed transient expression performed in co-transfection experiments and replication studies performed with HIV-1 infectious molecular clones (Kim et al. 1993; Zeichner et al. 1991). These studies indicated that alterations in LTR structure that result in lower or higher promoter activity in transient expression assays in a number of cell types resulted in decreases or increases in viral replication and infectious virus production in T cells and a number of other cell types, respectively. Other studies have indicated that the efficiency of viral gene transcription during the course of HIV-1 replication directly correlates with viral replication and infectious virus production (Garcia-Crespo et al. 2010). Cumulatively, these studies suggest that patient-derived HIV-1 LTR quasispecies with sequence alterations that yield lower or higher promoter activity would decrease or increase overall viral fitness.

Even though previous studies have shown that the 5T configuration of Sp site III or the 3T configuration of C/BEP site I is a knockout binding site for their cognate factors (Nonnemacher et al. 2004), we have demonstrated that a majority of patient-derived HIV-1 LTRs containing the 5T configuration of Sp site III or the 3T5T configuration exhibit basal transcription activity in the range of 0.5- to 2-fold over the HIV-1 LAI LTR basal activity (Fig. 3). These results suggested that the 5T or 3T5T sequence configurations did not lead to the production of a defective LTR structure but rather resulted in a spectrum of 5T- and 3T/5T-containing LTRs with an array of patient-derived LTR backbones. These patient-derived LTRs were responsive to Tat-mediated transactivation in Jurkat T cells and U-937 monocytic cells (Fig. 3). Furthermore, the results presented demonstrated that different patterns of basal transcription were observed in the two cell lines, suggesting that cellular phenotype may alter the functional properties of 5T- and/or 3T/5T-containing, patient-derived HIV-1 LTRs. This result is consistent with those from reports showing that HIV replication and LTR-mediated gene expression can be modulated by different transcription factors in a cell type-dependent manner (Moses et al. 1994).

Recent studies have demonstrated that brain-derived HIV-1 Tat clones from HIV-1 non-demented and demented (HIVD or HAD) individuals exhibit different transactivation activities with respect to LTR-directed transcription (Boven et al. 2007). HIV-1 LTR transactivation mediated by brain-derived Tat clones was examined by luciferase activity in human astrocytic cell line U-373MG and monocytoid cells (THP-1). Results demonstrated that Tat clones from non-demented individuals efficiently trans-activated the HIV-1 LTR,

whereas those from individuals with HAD were defective with respect to LTR-directed transactivation. The results reported here indicated that patient 107 presented neurologically normal at the R00 and R01 visits and later at R02 and R03 visits with neurocognitive decline (Table 2). In these studies, the full-length Tat clones derived from the PBMC compartments of patient 107 had transactivation ability and exhibited a little higher transactivation level than IIIB Tat101 in Jurkat T cells (Fig. 4). Taken together, the transactivation activity of patient 107 PBMC-derived Tat may also be associated with the patient's current neurocognitive performance. It has also been reported that the HIV-1 Tat protein may be co-selected with TAR variants (Marozzi et al. 1998). However, few studies have been performed with regard to the co-selection of HIV-1 Tat and LTR sequence variants. We have reported that both 107-R00 Tat101 and 107-R01 Tat101 have higher transactivation abilities than IIIB Tat101 with respect to patient 107-derived LTRs (107-R00 LTR and 107-R01 LTR). Furthermore, 107-R01 Tat101 could transactivate the colinear LTR (107-R01 LTR) to higher levels than 107-R00 Tat101, suggesting that a more efficient Tat is co-selected with the colinear LTR during the course of HIV disease. Sequence analysis revealed that a proline to serine change at position 6 may be responsible for this increased trans-activation potential. Preliminary molecular modeling studies suggest that this change may impact the interaction of Tat with the positive transcription elongation factor b (P-TEFb) complex (data not shown).

Overall, these results suggest that the 3T and 5T SNPs may be predictive of disease progression and the onset of neurocognitive impairment. Admittedly, there have been a number of reports that have demonstrated a waxing and waning pattern of HAND in their cohort (REF). Interestingly, within our cohort, we not only observe the "waxing and waning" patterns of HAND as has been reported but we also have observed varying amounts of specific viral quasispecies present in these patients. For example, we have identified 52 additional patients in the cohort that carry the 3T/5T variant as a minor species. Over time, this minor quasispecies can become more or less prevalent within a given patient. There are a number of factors that can contribute to which quasispecies is present and in what amount. These include antiretroviral therapy (ART) in that as a patient responds to ART the viral replication within different cell populations especially CD4⁺ T cell populations is decreased. Then as the patient stops responding to this therapy, replication begins to expand especially in this population of cells. It can be and has been hypothesized that different genetic variants may arise from different cell populations. The altered utilization of co-receptors over the course of HIV disease is an example of this. Another example that can add a layer of complexity to this concept is the stage of disease. As a patient progresses with disease, the total number of CD4⁺ T cells versus the number of cells of the monocyte-macrophage lineage changes. This of course occurs along with the "waxing and waning" patterns of viral load breakthrough due to lack of therapeutic response. Therefore, the ratio of quasispecies that comes from replication in these cell populations likely changes. Other factors such as use of drugs of abuse and opportunistic infections can also cause increases or decreases in HIV-1 gene expression, viral replication, and infectious virus production. All of these parameters likely play roles in altering the relative levels of specific viral quasispecies. Therefore, when we identify SNPs such as 3T and 5T or 3T/5T, we fully expect to potentially see them as the predominant genotype at certain times and not at other times.

The presentation of patient 107 and the 3T/5T genotype is the first attempt at identifying genotypes that once they are even a small portion of the quasispecies present can predict that a patient may experience an increase in specific levels of certain quasispecies based on changes in viral replication patterns in different cell populations that may ultimately lead to changes in the relative level of neurocognitive impairment in a given patient. Clearly, additional HIV-1-infected patients carrying the co-selected 3T/5T LTR SNP will need to be assessed in an ongoing longitudinal study to examine the prognostic value of this SNP with

respect to the development of HIV-1-induced neurocognitive impairment. In this regard, as mentioned above, a growing number of HIV-1-infected patients in the DREX-ELMED HIV/AIDS Genetic Analysis Cohort, including patient 107 (now numbering 52), have been shown to carry the 3T/5T co-selected SNP albeit at a low abundance level and are currently being followed utilizing 454 deep sequencing technology to quantitate the levels of specific SNPs in selected cell populations during the course of disease. These results also suggest that selective pressures may be exerted on the LTR, Tat, envelope, and perhaps other genes that can lead to the development of a viral swarm more or less capable of replicating in particular cells and contributing to neurological disease. Future studies will determine if other SNPs that correlate to disease parameters or neurological disease exist and will determine the relative fitness of patient-derived LTRs in multiple cell types with their colinear Tat and Env proteins to understand more fully the molecular mechanisms that drive HIV-1 disease. One could envision that in the future, as these studies continue to identify a panel of SNPs that correlate with and/or predict HAND when a patient's quasispecies spectrum is analyzed, it will be analyzed for the relative amount of selected SNPs present with the development of a risk calculation relating to the likelihood of developing some form of HAND.

Materials and methods

Patient enrollment, clinical data, and sample collection

Patients in the DREXELMED HIV/AIDS Genetic Analysis Cohort were recruited from the Partnership Comprehensive Care Practice of the Division of Infectious Disease and HIV Medicine at Drexel University College of Medicine (DREXELMED), located in Philadelphia, Pennsylvania. Patients infected with HIV-1 were eligible for enrollment into the study. The study was approved by the DREXELMED institutional review board. All subjects signed written informed consent. Once a patient was enrolled, his or her clinical data were collected and entered onto individual Case Report Forms with anonymous identifiers. Clinical information collected directly from the patient included age, year of birth, gender, ethnic group, race, HIV exposure category, tobacco and alcohol use history, illicit drug use history, potential pregnancy, and approximate year of seroconversion and mode of transmission. Additional clinical information was gathered directly from the patient chart, including initial, nadir, and current CD4⁺ T cell counts; initial, nadir, and current CD8⁺ T cell counts; peak and latest viral loads; disease status; current ART status; current ART regimen; past ART treatments; hepatitis B virus and hepatitis C virus co-infection; AIDS-defining illnesses; admission of illicit drug, alcohol, and tobacco; any notations on mental health history including inpatient stays for mental health reasons, any history of depression, schizophrenia, and other neurological diseases; and any notations on other complicating conditions related to HIV-1 infection. After the clinical information was collected and the neurological status of the patient was assessed (described below), blood samples (~50 mL) were drawn from each patient enrolled in the study. This was performed at the initial and each subsequent return visit. Blood samples were used for drug screening, serum analysis, and PBMC isolation (both procedures are described below). Each patient was called back for a longitudinal study approximately every 6 months, with at least one recall per year.

Neurological assessment

Each patient at each visit was also assessed for neurological impairment using a mini neurological bedside test (modified from the International HIV Dementia Scale; Sacktor et al. 2005). This test screens for short-term memory, concentration, and processing speed (Fig. 2a). The maximum score is 12, which correlates to no neurological impairment. Scores that are below 10 are considered to be indicative of neurocognitive impairment. To begin the

test, patients were given four common words in 4 s and were asked for immediate recall by the patient. The patient was then asked to recall the words 5 min after the start of the test. The patients were given one point for each word recalled spontaneously. For words not recalled, the patient was given a prompt, after which the patient was given 0.5 points per word recalled. The memory recall has a maximum score of four points. During the test, the patient's psychomotor speed was also assessed by instructing the patient to write the letters of the alphabet. If the patient was unable to write the alphabet, he or she was instructed to write the numbers 1 through 20. The patient scored six points when this task took <21 s, five points for between 21.1 and 24 s, four points for between 24.1 and 27 s, three points for between 27.1 and 30 s, two points for between 30.1 and 33 s, one point for between 33.1 and 36 s, and zero points for anything >36.1 s. The last component of this test assesses the patient's constructional ability by asking the patient to copy a three-dimensional drawing of a cube as quickly and precisely as possible, for a maximum of two points. The patient was scored two points when the cube is drawn in <25 s, one point when it is drawn between 25 and 35 s, and zero points for anything longer than 35.1 s. The patient also scored zero points if the drawing did not mimic a cube.

Patient drug screening

Blood samples were screened for a standard seven-drug profile, including amphetamines (amphetamine and methamphetamine); barbiturates (amobarbital, butalbital, pentobarbital, phenobarbital, and secobarbital); benzodiazepines (desalkylflurazepam, flurazepam, and diazepam); cannabinoids (THC and THCA); cocaine (cocaine and benzoylecgonine); opiates (codeine and morphine); and phencyclidine.

PBMC isolation

Three purple-top BD vacutainer tubes containing EDTA were used to collect whole blood from patients for serum and PBMC isolation. These samples underwent an initial centrifugation to isolate and collect serum from each patient. On average, approximately 9 mL of serum was collected per patient and placed in 1 mL aliquots at -85°C for long-term storage. Patient-derived PBMCs were then isolated from whole blood using Ficoll-hypaque (Amersham Biosciences, Piscataway, NJ, USA) density gradient centrifugation as described previously (Pirrone et al. 2010). Once isolated and sufficiently washed, the cells were counted, 5×10^6 cells were removed for genomic DNA extraction (Qiagen, Valencia, CA, USA), and the remainder of the cells were stored long term at -85°C .

Polymerase chain reaction amplification and sequencing of the HIV-1 LTR

From the genomic DNA isolated from each HIV-1-infected patient, PCR amplification was performed and the resultant LTR products were sequenced. The first round of PCR was completed with two primers that are specific for the HIV-1 LTR (forward: 5'-TGGAAGGGCTAATTCCTC-3', reverse: 5'-ACTGATTTTCCCAGACTCCCT-3'; Integrated DNA Technologies, Coralville, IA, USA) along with Phusion High-Fidelity Polymerase (New England BioLabs, Ipswich, MA, USA), deoxyribonucleoside triphosphates (Promega, Madison, WI, USA), and magnesium chloride. From this first round of PCR, 10 μL of the reaction was used to complete a second amplification step using nested primers specific for the HIV-1 LTR (forward: 5'-CACTCCCAACGAAGACAAGA-3', reverse: 5'-GAGGGATCTCTAGTTACCAG-3'; Integrated DNA Technologies) and conditions similar to those in the first round. A portion of the second round of PCR was then run on a 1% agarose gel, and the band corresponding to ~640 bps was quantitatively assessed for concentration by spot densitometry. Following quantitation, the PCR product was purified using ExoSAP-IT (USB Corp., Cleveland, OH, USA) and was subsequently sequenced (Genewiz, South Plainfield, NJ, USA). The overall LTR sequence for each patient was then analyzed for sequence variation throughout the

entire LTR, with particular focus on 12 predetermined binding sites (C/EBP site II, USF, Ets, Lef-1, ATF/CREB, C/EBP site I, NF- κ B site II, NF- κ B site I, Sp site III, Sp site II, Sp site I, and Oct I). The clinical and sequence information obtained from these studies was entered into a patient database along with patient information gathered from the drug testing report (LabCorp, Burlington, NC). All of this information was obtained at each longitudinal visit for all patients enrolled and recalled.

Analysis of PCR product sequencing results

ABI trace files analyzed from patients passed through the following criteria: (1) the raw trace files were processed using Phred (Brockman et al. 2008) and trimmed using a quality score of 20 as a threshold (P error=0.01%) and (2) sequences had a length >100 nucleotides. SNP identification was performed using the Mutation Surveyor package (SoftGenetics, State College, PA, USA). This software is unique in that it incorporates several options for working with hypervariable genomes. Sequences were aligned to the consensus B (Jan 2002, Los Alamos database) reference sequence. Both quality information from the trace files (Phred scores) and several statistical tests for identification and quality control of the called putative variations were used to identify a set of high-quality SNPs.

Cell line maintenance

The human T cell line Jurkat (American Type Culture Collection, Manassas, VA, USA, ATCC, TIB-152) and the human monocytic cell line U-937 (ATCC, CRL-1593.2) were grown in RPMI-1640 medium (Cellgro, Mediatech, Manassas, VA, USA). Medium for Jurkat cells was supplemented with 10% heat-inactivated fetal bovine serum (FBS; GemCell, West Sacramento, CA, USA), sodium bicarbonate (0.05%, Cellgro), and antibiotics (penicillin, streptomycin, and kanamycin at 0.04 mg/mL each, Cellgro). Media for U-937 and TF-1 cells were supplemented with 10% heat-inactivated FBS (Gem-Cell), antibiotics (penicillin and streptomycin at a concentration of 0.04 mg/mL each, Cellgro), glucose (4.5 g/mL, Cellgro), sodium pyruvate (1 mM, Cellgro), and HEPES (10 mM, Cellgro). All cells were maintained at 37°C in 5% CO₂ at 90% relative humidity.

Cloning of the 4.4-kb HIV-1 DNA fragment from HIV-1-infected patient PBMCs

From the genomic DNA isolated from PBMCs, PCR was used to amplify a 4.4-kb segment of the integrated HIV-1 genome corresponding to the 3' end of the proviral genome from the *vpr* gene to the end of the 3' LTR. This was accomplished using HIV-1 primers BA015 (5'-GAATGGAGGAGAAAGAGATATAGCACACAA-3') and BA044 (5'-TTACCAGAGTCACACAACAGAC-3'; Integrated DNA Technologies) in both rounds of amplification. Between rounds 1 and 2, PCR-amplified 4.4-kb fragments were gel-purified from 0.7% low-melt agarose gels. Gel samples corresponding to a molecular size of 4.4 kb were excised and melted, and 5 μ L was utilized in the second round of the PCR protocol. Following the second round of PCR, products were gel-purified from 0.7% agarose gels. Gel samples corresponding to a molecular size of 4.4 kb were excised and underwent gel extraction following the Promega gel extraction procedure (Promega). These PCR products were then incubated with dATP (Promega) and Taq polymerase to add an A overhang on the end of the purified DNA. This product was then cloned into the pCR4 TOPO vector using a TA cloning procedure as described by the manufacturer (Invitrogen, Carlsbad, CA). The cloned 4.4-kb fragments contained within the pCR4 TOPO vector were then transformed into Stbl3 competent cells (Stratagene, La Jolla, CA, USA). These were plated in Luria-Bertani (LB) agar plates and grown overnight at 37°C. Plates containing colonies were replica-plated and the HIV-1 sequence inserts were sequenced (Genewiz). The HIV-1 sequences obtained from the colonies were aligned and analyzed using Lasergene alignment software (DNASTAR, Madison, WI, USA). Once colonies that contained HIV-1 sequence were identified, they were picked and grown overnight in LB broth at 37°C. Plasmids

containing the 4.4-kb fragment of HIV-1 were isolated using a mini preparation procedure as described by the manufacturer (Qiagen).

Amplification of full-length *tat* from HIV-1 4.4-kb proviral DNA clone

A two-step PCR amplification procedure was performed to obtain *tat* exon I and II DNA. In the first step, the two exons were amplified in individual reactions utilizing the 4.4-kb HIV-1 proviral genomic fragment contained within the recombinant TA clones as template (primer F1 and R1 for exon 1 and primer F2 and R2 for exon 2, with primer R1 and F2 having an overlap region of 17 nucleotides). In the second step of the protocol, the two PCR products were ligated together using a mixture of the PCR products from the first step PCR amplification as template and primer F1 and R2 for amplification. Because of sequence variation within different patient-derived HIV-1 *tat* clones (especially between nucleotides 60 and 72 which exhibited the greatest degree of genetic variation), each *tat* required specific primers for PCR amplification. As one example, the sequence and amplification primers are shown for patient 107 clone 1 exon I and II.

107-c1 exon I

ATGGAGCCAGTAGATCCTAGACTAGAGCCCTGGAAGCATCCAGGAAGTCAGCC
TAAGACTGCTTGTACCCCTTGCTATTGTAAAAAATGCTGCTTTCATTGCCAAGTT
TGTTTCACAAAAAAGCCTTAGGCATCTCCTATGGCAGGAAGAAGCGGAGACA
GCGACGAAGAGCTCCTCAAGACAGTCAGACTGATCAAGGCTATCTAC**CAAAGC**
AG

107-c1-F1: 5'-GGGGTACC**ATGGAGCCAGTAGAT**

107-c1-R1: 5'-GGGGCTGGGCTGCTTTGGTAGATAG

107-c1 exon II

CCCAGCCCCAGCACCGAGGGGACCCGACAGGCCCGAAGGAATCGAAGAAGA
AGGTGGAGAGAGAGACAGAGGCAGGTCCGAAAGATTAG

107-c1-F2: 5'-CAAAGCAG**CCCAGCCCCAGCAC**

107-c1-R2: 5'-GGATATCCTAATCTTTCGGACC

The italicized nucleotides in the forward and reverse primers indicate the cleavage sites for the restriction endonucleases KpnI and EcoRV, respectively, which correspond to the sequence necessary for directional cloning into the expression vector pcDNA3.1(+)/Hygro (Invitrogen), described below. The bold nucleotides correspond to the nucleotides used as the portion of a traditional PCR primer, whereas the underlined nucleotides represent the overlap regions.

Plasmid construction and site-directed mutagenesis of HIV-1 LTRs and Tat

The HIV-1 LAI LTR (approximately 640 bp) was derived from the HIV-1_{LAI} molecular clone of HIV-1 by PCR amplification using the forward primer: 5'-GGGGTACCTGGAAGGGCTAATTC**ACTCC**-3' and reverse primer: 5'-TCCCCGGGTGCTAGAGATTTTCCACA-3' (Integrated DNA Technologies). The italicized nucleotides indicate the restriction endonuclease cleavage sites KpnI and SmaI, respectively. The amplified product was digested with KpnI and SmaI (Promega) and ligated into a modified pGL3-Basic vector, which contained the firefly luciferase (Luc) gene (Promega), to construct the parental HIV-1-LAI-LTR-Luc expression construct. The

parental construct was used as a template for site-directed mutagenesis using the Quick Change mutagenesis procedure (Stratagene) to generate the mutant construct LAI-LTR-SpIII-5T. The following primers were used for site-directed mutagenesis; the nucleotide that was mutated is underlined. Forward primer: 5'-CTTTCCAGGGAGGTGTGGCCTAGGCAG-3'; reverse primer: 5'-CTGCCTAGGCCACACCTCCCTGGAAAG-3' (Integrated DNA Technologies).

Construction of patient-derived HIV-1 LTR-driven luciferase vectors was performed as follows. First, the patient-derived LTRs were amplified from their 4.4-kb clones using forward primer LL8-conB: 5'-GGGGTACCTGGAAGGGCTAATTTACTTCC-3' and reverse primer LL7-conB: 5'-TCCCCCGGGCTAATTTACTCC-3'. The italicized nucleotides indicate the restriction endonuclease cleavage sites KpnI and SmaI, respectively. Subsequently, the pGL3-Basic vector (Promega) and LTR PCR products were digested with KpnI and Xma I (Promega). Once digested, the LTRs were ligated into the pGL3 vector and transformed into DH5 α competent cells. Colonies were selected and DNA plasmids were obtained utilizing a mini preparation procedure as described by the manufacturer (Qiagen) and sequenced to confirm the maintenance of the original LTR sequence (Genewiz). Sequences were analyzed using Lasergene software (DNASTAR). Once the sequence was confirmed, a large-scale plasmid DNA preparation was used as described by the manufacturer (Qiagen) for use in transient transfection experiments.

The patient-derived *tat* sequences amplified above and the pcDNA3.1(+)/Hygro expression vector were digested with KpnI and EcoRV. Once digested, the Tats were ligated into the pGL3 vector and transformed into DH5 α competent cells. Colonies were selected and DNA plasmids were obtained using a mini preparation procedure as described by the manufacturer (Qiagen) and sequenced to confirm the maintenance of the original Tat sequence (Genewiz). Sequences were analyzed using Lasergene software (DNASTAR). Once the sequence was confirmed, a large-scale plasmid DNA preparation was used as described by the manufacturer (Qiagen) for use in transient transfection experiments.

Transient expression analyses

Exponentially growing Jurkat T cells and U-937 cells (1×10^6) were seeded in 2 mL of growth medium in six-well plates. For each transfection, FuGene 6 transfection reagent (6 μ L) (Roche, Indianapolis, IN, USA) was dispensed into serum-free medium (94 μ L). After 5 min, the solution was added to the DNA dropwise, incubated for 15 min, and dispensed dropwise into the cell culture. Cells were transfected with 1,000 ng of firefly luciferase LTR expression constructs (LAI-LTR, or LAI-5T or patient-derived LTRs) in conjunction with 50 ng pRL-TK Renilla luciferase internal control vector. When co-transfection with 500 ng of HIV-1 IIIB Tat or patient-derived Tat expression vector DNA was performed, the same amount of pcDNA3.1 empty vector DNA was included in the FuGene6-DNA reaction in the absence of Tat plasmid DNA. Cells were harvested 24 h after transfection, and cell extracts were assayed using the Dual Luciferase Assay system as described by the manufacturer (Promega). Firefly luminescence was normalized to Renilla luminescence to control for variability in transfection efficiency. Firefly luminescence in pGL3 constructs was presented with the parental HIV-1 LAI LTR activity equivalent to 1.0 for each experiment, and the relative fold activity of the mutagenized or the patient-derived LTR constructs was normalized relative to this value. Each experiment was performed in triplicate with a representative experiment shown. The error bars shown for each data point indicate the standard deviation from one representative experiment run in triplicate.

In silico HIV-1 co-receptor use prediction

The *Env* DNA sequence from the 4.4-kb clones was translated using the Lasergene software package (DNASTAR). The amino acid sequence corresponding to the V3 loop of gp120 was then analyzed using multiple prediction tools. First, the amino acid sequence was analyzed for the amino acid present at positions 11 and 25 of the V3 loop. The aptly named 11/25 rule refers to charges of V3 residues 11 and 25: If one of these residues contains a positive charge, then the prediction is that the virus is CXCR4-using (X4), whereas the absence of a positive residue at either of these positions indicates a virus that is CCR5-using (R5; de Jong et al. 1992; Fouchier et al. 1992; Shioda et al. 1992). Second, the V3 portion of the envelope sequence was also analyzed for net charge by the ProtParam Tool (<http://www.expasy.ch/tools/protparam.html>). ProtParam (Gasteiger et al. 2005) is a tool provided by ExPASy, a bioinformatics program used for protein analysis that calculates several physicochemical properties such as molecular weight, theoretical pI, amino acid composition, atomic composition, extinction coefficient, estimated half-life, instability index, aliphatic index, and grand average of hydropathicity (GRAVY) that can be determined directly from protein sequence without any other additional information. If the net charge is +6 or above, it is designated as X4-utilizing and if +5 or below is designated as R5-utilizing. Third, the amino acid sequence was analyzed by the Geno2Pheno (co-receptor) program (<http://coreceptor.bioinf.mpi-inf.mpg.de/index.php>), an HIV-1 co-receptor usage prediction tool that has been developed from the support vector machine statistical learning method. The strategy of co-receptor classification is based on the ability of virus to use CXCR4. Therefore, the dual-tropic (X4R5) virus is identified as X4-utilizing. The prediction evaluation relies mainly on the V3 sequences from the Los Alamos HIV sequence database (<http://www.hiv.lanl.gov/>) and other public data. This method used 1100V3 sequences (769 R5, 210 X4, 131 R5X4) from 332 patients to generate its prediction algorithm. The degree of CXCR4 usage prediction conservation can be set according to the purpose. In addition, this method uses additional clinical parameters [viral load, CD4⁺/CD8⁺ cell counts and CD4 percentages (nadir counts for all three), and CCR5 genotype] to improve the prediction performance. Finally, the PSSM (Position-Specific Scoring Matrices) program was used (<http://indra.mullins.microbiol.washington.edu/webpssm/>). PSSM (Jensen et al. 2003; Jensen and van't Wout 2003) is a bioinformatics tool designed for HIV-1 co-receptor usage prediction based on the 35 amino acid sequence of the V3 loop. The V3 sequence is first aligned to the conB sequence using the Needleman–Wunsch algorithm and amino acid distance matrix. There are two matrices for scoring for subtype B viral sequences: X4R5 and SINSI. The X4R5 matrix calculates using sequences of known co-receptor phenotype, as assayed on indicator cells expressing exogenous CD4 and either CCR5 or CXCR4. The SINSI matrix is calculated using sequences of known syncytium-inducing phenotype on the MT2 cell line.

Statistical analysis

The results were statistically analyzed by the two-sided Student's *t* test. Differences between groups were considered significant if *P* values <0.05 were obtained.

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ConB TGGAGGGCTAAATTACTCCAAAAAGACAAGATATCCTTGATCTGTGGGTCTACACACACAAGGCTACTTCCCTG
 A0107-R00 -----NGGTNATN-TTNCCGCTTCCACNCTTTTGGCTCAATTCCTG
 A0107-R01 -----AAATCTTGATTGTGGGTTCACACACAAGGCTACTTCCCTG
 A0107-R02 -----ANNTCTGATTGNGGGTTTCCACACACAANGCTACTTCCCTG
 A0107-R03 -----

ConB ATTGGCAGAACTACACACAGGGCCAGGGTCAGATATCCACTG--ACCTTTGGATGGTGC-TTCAAGTAGTACCAG
 A0107-R00 ATTGGCAGAACTACACACAGGGCCAGGGTCAGATATCCACTG--ACCTTTGGATGGTGC-TTCAAGTTANTACCAG
 A0107-R01 ATTGGCAGAACTACACACAGGGCCAGGGTCAGATATCCACTG--ACCTTTGGATGGTGC-TTCAAGTTAGTACCAG
 A0107-R02 ATTGGCAGAACTACACACAGGGCCAGGGTCAGATATCCACTG--ACCTTTGGATGGTGC-TTCAAGTTAGTACCAG
 A0107-R03 ATTGGCAGAACTACACACAGGGCCAGGGTCAGATATCCACTG--ACCTTTGGATGGTGC-TTCAAGTTAGTACCAG
 *****

ConB TTGAGCCAGAGAAGGTAGAAGAGGCCAATGAAGGAGAGAACAACAGCTTGTACACCTATGAGCTGCATGGGATGG
 A0107-R00 TTGAGCCAGANAAGGTAGAAGAGGCCAATGAAGGAGAGAACAACAGCTTGTACACCTATGAGCCAGCATGGAATGG
 A0107-R01 TTGANCCAGATAAGGTAGAAGAGGCCAATGAAGGAGAGAACAACAGCTTGTACACCTATGAGCCAGCATGGAATGG
 A0107-R02 TTGANCCAGATAAGGTAGAAGAGGCCAATGAAGGAGAGAACAACAGCTTGTACACCTATGAGCCAGCATGGAATGG
 A0107-R03 TTGAGCCAGATAAGGTAGAAGAGGCCAATGAAGGAGAGAACAACAGCTTGTACACCTATGAGCTGCATGGAATGG
 * * * * *

ConB C/EBP II USF Ets →
 A0107-R00 AGGACCCGAGAAAAGTGTAGTGTGAAGTTTGACAGCCGCTAGCATTTCATCACATGGCCGAGAGCTGCATC
 A0107-R01 ATGACCCANAGAAAAGTNTTGCAGTGAAGTTTGACAGCCGCTAGCATTTCATCACATGGCCGAGAGCTGCATC
 A0107-R02 ATGACCCAGAGAAAAGTGTAGTGTGAAGTTTGACAGCCGCTAGCATTTCATCACATGGCCGAGAGCTGCATC
 A0107-R03 ATGACCCAGAGAAAAGTGTAGTGTGAAGTTTGACAGCCGCTAGCATTTCATCACATGGCCGAGAGCTGCATC
 * * * * *

ConB Lef-1 ATF/CREB C/EBP I NF-κB II NF-κB I Sp III
 A0107-R00 CGGAGTACTACAAGGACTGCTGACATCGAGCTTTCTACAAGGGACTTCCGCTGGGGACTTTCCAGGGAGGCTGGCC
 A0107-R01 CGGAGTACTACAAGGACTGCTGACATCGAGCTTTCTACAAGGGACTTCCGCTGGGGACTTTCCAGGGAGGCTGGCC
 A0107-R02 CGGAGTACTACAAGGACTGCTGACATCGAGCTTTCTACAAGGGACTTCCGCTGGGGACTTTCCAGGGAGGCTGGCC
 A0107-R03 CGGAGTACTACAAGGACTGCTGACATCGAGCTTTCTACAAGGGACTTCCGCTGGGGACTTTCCAGGGAGGCTGGCC
 *****

ConB Sp II Sp I Oct I
 A0107-R00 TG-GGCGGACTGGGGAGTGGCGAGCCCTCAGATGCTGCATATAAGCAGCTGCTTTTGGCTGTACTGGGTCTCTCTG
 A0107-R01 TG-TNGCGGNANTGGGGAGTGGCGAGCCCTCAGATGCTGCATATAAGCAGCTGCTTTTGCCTGTACTGGGTCTCTCTG
 A0107-R02 TG-GGCGGACTGGGGAGTGGCGAGCCCTCAGATGCTGCATATAAGCAGCTGCTTTTGCCTGTACTGGGTCTCTCTG
 A0107-R03 TG-GGCGGACTGGGGAGTGGCGAGCCCTCAGATGCTGCATATAAGCAGCTGCTTTTGCCTGTACTGGGTCTCTCTG
 * * * * *

ConB GTTAGACCAGATCTGAGCCTGGGAGCTCTCTGGCTAACTAGGGAACCCACTGCTTAAGCCTCAATAAAGCTTGCCCTTG
 A0107-R00 GTTAGACCAGATCTGAGCCTGGGAGCTCTCTGGCTAGCTAGGGAACCCACTGCTTAAGCCTCAATAAAGCTTGCCCTTG
 A0107-R01 GTTAGACCAGATCTGAGCCTGGGAGCTCTCTGGCTAGCTAGGGAACCCACTGCTTAAGCCTCAATAAAGCTTGCCCTTG
 A0107-R02 GTTAGACCAGATCTGAGCCTGGGAGCTCTCTGGCTAGCTAGGGAACCCACTGCTTAAGCCTCAATAAAGCTTGCCCTTG
 A0107-R03 GTTAGACCAGATCTGAGCCTGGGAGCTCTCTGGCTAGCTAGGGAACCCACTGCTTAAGCCTCAATAAAGCTTGCCCTTG
 *****

ConB AGTGCTTCAAGTAGTGTGCCCCGTCTGTTGTGTGACTCTGGTAAGTACAGATCCCTCAGACCTTTTAGTCAGTGTG
 A0107-R00 AGTGCTTAAAGTAGTGTGCCCCGTCTGTTGTGTGACTCTGGTAAGTACAGATCCCTCAGACCTTTTNNANNTCAAN
 A0107-R01 AGTGCTTAAAGTAGTGTGCCCCGTCTGTTGTGTGACTCTGGTAAGTACAGATCCCTCAACNTTTTTTNNNNAAA
 A0107-R02 AGTGCTTAAAGTAGTGTGCCCCGTCTGTTGTGTGACTCTGGTAAGTACAGATCCCTCAACTTTTTTTA-----
 A0107-R03 AGTGCTTAAAGTAGTGTGCCCCGTCTGTTGTGTGACTCTGGTAAGTACANNATCCCTCA-----
 *****

ConB GAAAACTCTAGCA
 A0107-R00 AAA-----
 A0107-R01 ANAAAA-----
 A0107-R02 -----
 A0107-R03 -----

Fig. 1.

Sequence alignment of all patient 107 visits and identification of specific transcription factor binding sites. PCR-amplified HIV-1 LTRs from all visits of patient 107 were sequenced and aligned to the conB HIV-1 LTR (Jan 2002) as described in “Materials and methods.” SNP identification was performed using the mutation surveyor package (SoftGenetics) throughout the entire LTR, with particular focus on 12 predetermined binding sites (C/EBP site II, USF, Ets, Lef-1, ATF/CREB, C/EBP site I, NF-κB site II, NF-κB site I, Sp site III, Sp site II, Sp site I, and Oct I), indicated by a line above the appropriate sequence. An asterisk indicates nucleotide positions from all visits that align to conB

Fig. 2. Patient 107 acquires neurocognitive impairment as assessed by the mini bedside neurological exam. **a** The mini bedside neurological exam was modified from the International HIV Dementia Scale (Sacktor et al. 2005). This test screens for short-term memory as well as for concentration and processing speed as described in “Materials and methods.” **b** Patient 107 was assessed at each visit for neurological impairment using the mini neurological bedside test. Scores for each visit are presented along with a scan of the cube drawn at each visit

Fig. 3. Patient-derived HIV-1 LTRs containing a 5T Sp site III exhibit a broad spectrum of basal and Tat-mediated transcription activity in Jurkat T cells (**a**) and U-937 monocytic cells (**b**). Patient-derived LTR expression constructs (1,000 ng) plus and minus HIV-1 IIIB Tat86 expression vector (500 ng) were transiently transfected into 10^6 Jurkat T cells (**a**) or U-937 monocytic cells (**b**) using the FuGene 6 transfection reagent, in parallel with luciferase reporter constructs driven by the HIV-1 LAI LTR and the LAI LTR containing the 5T variant. At 24 h post-transfection, LTR basal and Tat-mediated transcription activity was detected by the Dual-Luciferase reporter assay; results were presented as the average fold over LAI basal activity in three independent experiments. Patient-derived HIV-1 LTRs containing the 5T Sp site III induced by IIIB Tat presented as the average fold over their own basal activity are presented *above each graph*. Representative results shown are derived from transfection performed in triplicate. Mean values ($\pm$ SD) for each experimental result are shown. An *asterisk* denotes a statistically significant difference in activity compared to the LAI LTR basal activity with $P < 0.01$

Fig. 4.

HIV-1-infected patient 107 Tat transactivates its colinear patient-derived LTR better than an unmatched Tat protein in Jurkat T cells. LAI or HIV-1-infected patient 107-derived LTR expression construct (1,000 ng) and expression vectors (500 ng) containing IIIIB Tat101, HIV-1-infected patient 107-R00-Tat, or 107-R01-Tat were co-transfected into Jurkat T cells. LTR basal and Tat-mediated transcriptional activity was determined 24 h post-transfection by the Dual-Luciferase reporter assay; results were presented as the average fold over LAI basal activity. Representative results shown are derived from transfection experiments performed in triplicate. Each experimental result is presented as a mean value ($\pm$ SD). An *asterisk* denotes a statistically significant difference in activity compared to Tat-transactivated LAI LTR with $P < 0.05$. *Double asterisks* denote a statistically significant difference in activity compared to Tat-transactivated LAI LTR with $P < 0.01$.

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ConB      MEPVDPRLPEPWKHGPSQPKTACTNCYCKKCCFHCQVCFITKGLGISYGRKK
IIIB      MEPVDPRLPEPWKHGPSQPKTACTNCYCKKCCFHCQVCFITKALGISYGRKK
107-R00   MEPVDPRLPEPWKHGPSQPKTACTPCYCKKCCFHCQVCFITKKALGISYGRKK
107-R01   MEPVDSRLPEPWKHGPSQPKTACTPCYCKKCCFHCQVCFITKKALGISYGRKK
          *****
ConB      RRQRRRAPQDSQTHQVSLSKQPASQPRGDPGPKESKKKVERETETDPVD
IIIB      RRQRRRPPQGSQTHQVSLSKQPTSQSRGDPGPKESKKKVERETETDPFD
107-R00   RRQRRRAPQDSQTDQGYLPKQPSQPHRGDPGPKESKKKVERETEAGPKD
107-R01   RRQRRRAPQDSQTDQGYLPKQPGPQHRGDPGPKESKKKVERETEAGPKD
          *****

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Fig. 5.

Patient-derived HIV-1 Tat clones from patient 107 have non-consensus variations compared with IIIB or conB Tat. HIV-1-infected, patient-derived Tat clones from patient 107 were obtained from the 4.4-kb cloned fragments with their colinear LTRs. Sequence alignments of patient-derived Tat proteins were compared with those from the laboratory strain IIIB and conB Tat. An *asterisk* indicates an amino acid residue from all visits that align to the conB Tat. A *dot* indicates conservation of weak groups. A *colon* indicates conservation of strong groups. A *space* indicates non-consensus changes among the compared residues

Table 1

Single nucleotide polymorphisms in the 12 transcription factor binding sites of patient 107

Patient visit	C/EBP site II	USF	Ets	Lef-1	ATF/CREB	C/EBP site I	NF-κB site II	NF-κB site I	Sp site III	Sp site II	Sp site I	Oct I
A0107-R00	ConB	ConB	1A	7A 12A	ConB	3T	ConB	ConB	ConB	ConB	ConB	3C
A0107-R01	ConB	ConB	1A	7A 12A	6C	3T	ConB	ConB	5T	ConB	ConB	3C
A0107-R02	ConB	ConB	1A	7A 12A	6C	3T	ConB	ConB	5T	ConB	ConB	3C
A0107-R03	ConB	4G	ConB	2T 7A	ConB	3T 6G	ConB	ConB	ConB	ConB	ConB	ConB

Sequence variation in each transcription factor binding site from the sequenced LTR PCR product from patient 107 was determined by comparison with the last consensus subtype B (conB) LTR determined in Jan of 2002 by the Los Alamos HIV database (<http://www.hiv.lanl.gov/>). The variant is designated by the nucleotide position in that particular binding site and the nucleotide it is changed to. For example, if position 3 of C/EBP site 1 has a C to T change, it is designated as a 3T variant. If there are no changes, it is designated as conB

Disease severity of patient 107 increases over the four visits

Table 2

Patient visit	Age	Yrs seropositive	Nadir CD4	Current CD4	Peak viral load	Current viral load	Number of AIDS-defining illnesses	Hepatitis B status	Hepatitis C status	HAART status	Admitted drugs of abuse status	Drugs of abuse tested positive for
R00 (visit 1)	43	3	95 (162)	565	455,000		0	ND	ND	cH	Deny any use	Test negative
R01 (visit 2)	44	4	95 (162)	659	455,000		1	Negative	Negative	cH	Admit past use	Test negative
R02 (visit 3)	44	5	95 (162)	720	455,000		1	Negative	Negative	cH	Admit past use	Test negative
R03 (visit 3)	45	6	95	95	455,000		1	Negative	Negative	cH	Admit past use	Test ND

Clinical parameters for patient A0107 were collected verbally or through a review of the medical record. Age is presented in years; nadir and current CD4⁺ T cell count in cells per microliter; peak and current viral load in copies per milliliter; and haart status as continuous (cH) or discontinuous (dH). The nadir CD4⁺ T cell count presented in parentheses was the nadir for the patient at all visits until R03 where it dropped to 95 cells/ μ L.

ND not determined

Table 3

Co-receptor use prediction using in silico prediction methods

Patient visit	Clone no.	Sequence observational analysis		ProtParam (ExPASy)		Geno2pheno		PSSM		Percentile	X4/R5 (sinsi)
		11-25	11-25 prediction	Net charge	Prediction	Prediction	X4 or R5	Genotype	Pos charge	Net charge	
R00	1	11R	X4	+7	X4	X4	X4	RR	9	+8	0.61
		25R									X4
R01	1-11	11S	R5	+4	R5	R5	R5	SA	7	+6	0.48
		25A									R5
R02	1	11S	X4	+5	R5	R5	R5	SA	8	+7	0.77
		25A									R5
	2	11R	R5	+7	X4	X4	X4	RA	9	+8	0.96
		25A									X4
	3	11S	R5	+4	R5	R5	R5	SA	7	+6	0.48
		25A									R5
R03	1-10	11G	R5	+2	R5	R5	R5	GD	7	+3	0.96
		25D									R5