Lentiviral LTR-directed Expression, Sequence Variation, and Disease Pathogenesis

Fred C. Krebs[1], Tricia H. Hogan[1], Shane Quiterio[1], Suzanne Gartner[2], and Brian Wigdahl [1*]

[1]The Pennsylvania State University, College of Medicine, Department of Microbiology and Immunology, Hershey, Pennsylvania 17033, USA

[2]Johns Hopkins School of Medicine, Department of Neurology, Johns Hopkins University, Baltimore, Maryland 21218 USA

[*]Corresponding author
Mailing address: The Pennsylvania State University College of Medicine, Department of Microbiology and Immunology, 500 University Drive, P.O. Box 850, Hershey, PA 17033
Tel. (717) 531-8258 Fax. (717) 531-5580
E-mail: bwigdahl@psu.edu Web site: www.hmc.psu.edu/wigdahl

I. Introduction

The Lentivirus genus, itself a subset of the *Retroviridae* family of RNA viruses, includes viruses that share a common replicative cycle, which is regulated by key events throughout the cycle. One such event in the viral life cycle is the expression of the viral genome regulated by the activity of the long terminal repeat (LTR). The LTR serves as a convergence point for transcription factors and elements of the transcriptional machinery from the host cell as well as virus-encoded proteins that enhance or modulate LTR activity and the subsequent expression of viral RNA and proteins. LTRs contain elements that interact with the basic transcriptional machinery of the host cell, transcription factors that are found in a wide range of cell types susceptible to infection, factors expressed in a cell-type specific pattern or a select subset of cells, and factors expressed in conjunction with cellular differentiation, activation, and progression through the cell cycle.

Infections by each of the lentiviruses, including the human immundeficiency virus types 1 and 2 (HIV-1 and HIV-2), simian immunodeficiency virus (SIV), feline immunodeficiency virus (FIV), visna virus, equine infectious anemia virus (EIAV), and caprine arthritis/encephalitis virus (CAEV), cause cellular and systemic events that initiate disease processes. While bovine immunodeficiency virus (BIV) infection does not appear to result in a clinical disease, it is nevertheless classified as a lentivirus based on its genomic organization and biological properties. Pathophysiologic events associated with each of these viruses (principally immunologic and neurologic deficiencies) require that the virus propagate efficiently in cell types able to support infection, including macrophages, which serve as hosts in all lentiviral infections. Productive progression through the viral replication cycle requires sufficient expression of viral regulatory and structural genes, which is in turn dependent on adequate function of the LTR within cells hosting the infection. LTR function has a direct impact on (i) the productive infection of the host cell, (ii) viral expression and assembly sufficient to drive viral loads to levels associated with pathogenesis and disease progression, (iii) the transition from a latency-like level of viral expression to a highly productive infection, (iv) production of cytotoxic viral proteins, and (v) production of sufficient virus for dissemination to compartments throughout the body, including the peripheral circulation, lymphatic system, brain, and lungs.

This review focuses on lentiviral LTRs, regulation of their function, and their roles in disease processes associated with viral infection. Following a brief overview of HIV-1, HIV-2, and SIV pathogenesis, subsequent sections will describe studies that have expanded our understanding of (i) structure/function relationships between the HIV-1 LTR and immunopathogenesis, neuropathogenesis,

and quasispecies development, (ii) LTR function in the context of HIV-2-associated pathogenesis, (iii) the SIV LTR and disease processes associated with SIV infection of experimental animals, and (iv) insights gained from studies of other lentiviral LTRs, including FIV, BIV, visna virus, EIAV, and CAEV.

II. Lentiviruses as agents of pathogenesis

Four events are recognized as milestones in the field of human retrovirus research: the discovery of the human T cell leukemia virus type I (HTLV-I) in 1980 (1), the clinical recognition of the acquired immunodeficiency syndrome (AIDS) in 1981 (2), the isolation of what was later known as HIV-1 in 1983 (3), and the establishment of HIV-1 as the causative agent of AIDS (4,5). The investigations that followed the discovery of human retroviruses, the recognition of retroviruses as agents of neoplasia, immunodeficiency, and neuropathogenesis, and the global expansion of the AIDS epidemic have resulted in an extensive body of literature concerning HIV-1 and other retroviruses. The following provides a brief overview of disease processes associated with HIV-1, HIV-2, and SIV. Numerous reviews, including several pertinent chapters in *Fields Virology* (6-9) and many overviews focused on specific aspects of virology and pathogenesis, provide more detailed information (10-17).

A. Human immunodeficiency virus type 1 (HIV-1)

The most widely recognized consequence of HIV-1 infection is the slow and progressive deterioration of the integrity and function of the immune system, which is a characteristic shared by HIV-2, SIV (during infection of macaques), and FIV. Although the clinical presentation of HIV-1-associated immune system dysfunction varies from individual to individual, the infection, without therapeutic intervention, generally unfolds in three phases over a period of approximately eight to ten years after initial exposure to the virus: acute infection (lasting approximately three months), clinical latency (lasting typically eight to ten years), and clinically apparent disease (lasting two to three years) (18). The acute phase is characterized by a large, but transient viremia within the peripheral circulation and sometimes an acute mononucleosis-like syndrome. During clinical latency, there is a slow but steady decline in the number of CD4-positive T lymphocytes and in the general integrity of the immune system, a very low level of detectable virus within the peripheral blood, and a concurrent decrease in the titer of antiviral antibodies (18). During this period, HIV-1 replication is readily detectable in the lymph nodes despite almost undetectable levels of plasma virus (18-20). The clinically apparent phase of disease is typified by the reappearance of HIV-1 viremia, a precipitous loss of CD4-positive T lymphocytes, a rapid decline in immune function, and numerous AIDS-defining illnesses (7). Without pharmacotherapeutic intervention, the ultimate outcome of this phase is death for a vast majority of infected individuals.

As more HIV-1-infected patients were identified and diagnosed, an association between HIV-1 infection and the emergence of CNS pathologic and functional abnormalities became recognized. The spectrum of neurologic abnormalities associated with nervous system infection was collectively referred to as the AIDS dementia complex (ADC) or, more recently, HIV-1 dementia (HIVD). Approximately 30% to 60% of all HIV-1-infected individuals exhibit symptoms characteristic of HIVD (21), including dementia with progressive loss of cognitive functions, decreased memory, difficulty concentrating, psychomotor retardation, headaches, motor deficits, seizures, and psychiatric dysfunction. Although HIVD may appear at any time during the progression of HIV-1 infection and is not necessarily temporally linked to the progression of systemic immunologic dysfunction, it most often appears late in the course of disease concurrent with low CD4-positive cell numbers and high viral titers in the peripheral circulation (22).

B. Human immunodeficiency virus type 2 (HIV-2)

While HIV-2 is genetically more closely related to SIV than to HIV-1 (suggesting a past cross-species transmission event) (17,23), comparisons between HIV-2 and HIV-1 are nevertheless enlightening because of considerable differences in pathogenesis despite similarities in genomic structure and virology. Epidemiologic studies have shown that the majority of patients infected with HIV-2 are

asymptomatic, although a small percentage of patients present with an immunodeficiency indistinguishable from AIDS (24-26). In that patient subset, infection leads to immunodeficiency characterized by a decline in CD4-positive T cells, the emergence of opportunistic infections, and, ultimately, death (17). In contrast to HIV-1, HIV-2 infection is characterized by considerably lower viral loads independent of the duration of the infection, suggesting a link between reduced plasma viremia and decreased pathogenesis (27). But despite the spectrum of clinical disease progression, HIV-2-infected individuals survive longer than HIV-1 patients (28), with the course of disease extending to at least 16 years (29). Although some isolates of HIV-2 are associated with AIDS, others may be far less pathogenic, and HIV-2 as a group is less pathogenic than HIV-1 (24,26,29).

C. Simian immunodeficiency virus (SIV)

The term SIV refers to a family of lentiviruses able to infect numerous species of African primates. SIV infection of Asian macaques, especially with the highly pathogenic SIV_{sm} and SIV_{mac} strains, results in the appearance of an immunodeficiency similar to the disease associated with HIV-1 infection of the human immune system (16). This characteristic has made the combination of SIV and macaques a very attractive and useful animal model for the study of diseases associated with HIV-1 infection, for the development and testing of potential vaccines for HIV-1, and for evaluating pharmacotherapeutic approaches to treating HIV-1-infected patients. The utility of SIV as an animal model for HIV-1 is strengthened by strong similarities between both viruses, including genomic organization, mechanisms of viral gene expression, and tropism for CD4-positive T lymphocytes and macrophages (16).

Like the course of infection taken by HIV-1, the disease process initiated by SIV can be divided into three phases: the primary infection, the asymptomatic phase, and the late phase (AIDS) (16). However, in contrast to HIV-1, the span of this process is only one to two years in rhesus macaques and approximately 90 days in pig-tailed macaques. The primary infection, which lasts approximately three weeks post-inoculation, is characterized by the appearance of a high viral load, humoral and cellular immune responses, and clinical presentations including fever, diarrhea, anorexia, and malaise (30,31). During the asymptomatic phase, the animals appear healthy, despite lymphadenopathy and a steady decline in circulating T cells. The final phase, with the onset of severe CD4-positive T cell depletion and the appearance of numerous opportunistic infections, is very characteristic of AIDS caused by HIV-1.

As in HIV-1-infected patients, many SIV-infected macaque monkeys develop lentivirus-induced encephalitis (SIV encephalitis, SIVE). Macaques infected with SIV display neuropathologic features similar to those observed in HIVD patients, including gross subcortical cerebral atrophy, multinucleated giant cells, white matter lesions, and microglial nodules. SIV-infected macaques with CNS involvement also exhibit cognitive and motor deficits similar to those observed in humans with HIVD (32). Simian models of HIV-1 neuropathogenesis include pig-tailed macaques infected with the neurovirulent strain SIV/17E-Fr (33). Recent studies that combine the SIV/17E-Fr strain with the immunosuppresive SIV/DeltaB670 swarm of dual-tropic viruses have generated a disease model characterized by a rapid progression to AIDS, a high frequency of CNS lesions, and a short disease course (34). Neuronal dysfunction in macaque monkeys infected with SIV is primarily the result of macrophage/microglial infection, which is also certainly a contributor to the development of HIV-1-associated dementia in humans.

III. Lentiviral LTR structure/function and disease pathogenesis

Viruses of the Retrovirus family, including the lentiviruses HIV-1, HIV-2, SIV, BIV, FIV, and visna virus, share common physical and genomic traits, as well as a common replication cycle, which is characterized by key events, including viral binding and entry, reverse transcription of the RNA genome, integration of the resulting proviral DNA into the host genome, viral gene expression, virus assembly, and progeny virus budding and maturation. The LTR is integrally involved in several of these events and has its origins in the unique 5′ (U5) and 3′ (U3) regions as well as the flanking R regions of the single-stranded RNA genome. The multi-step process of reverse transcription results in the placement of two identical

LTRs, each comprised of a U3, R, and U5 region, at either end of the proviral DNA. The ends of the LTRs subsequently participate in integration of the provirus into the host genome. Once the provirus has been integrated, the LTR on the 5′ end serves as the promoter for the entire retroviral genome, while the LTR at the 3′ end provides for nascent viral RNA polyadenylation and, in HIV-1, HIV-2, and SIV, encodes the accessory protein, Nef.

A. Physical structure of the HIV-1 LTR and involvement in pathogenesis

The HIV-1 LTR is approximately 640 bp in length and, like other retroviral LTRs, is segmented into the U3, R, and U5 regions. The U3 region has been further subdivided according to transcription factor sites that populate the LTR and their impact on LTR activity and viral gene expression (Fig. 1). Numerous reviews have documented the growing list of transcription factors that interact with the LTR, their respective binding sites, and other viral and cellular factors involved in the regulation of HIV-1 LTR activity (35-39). The key landmarks of the core region are the TATAA box and the GC-rich binding sites

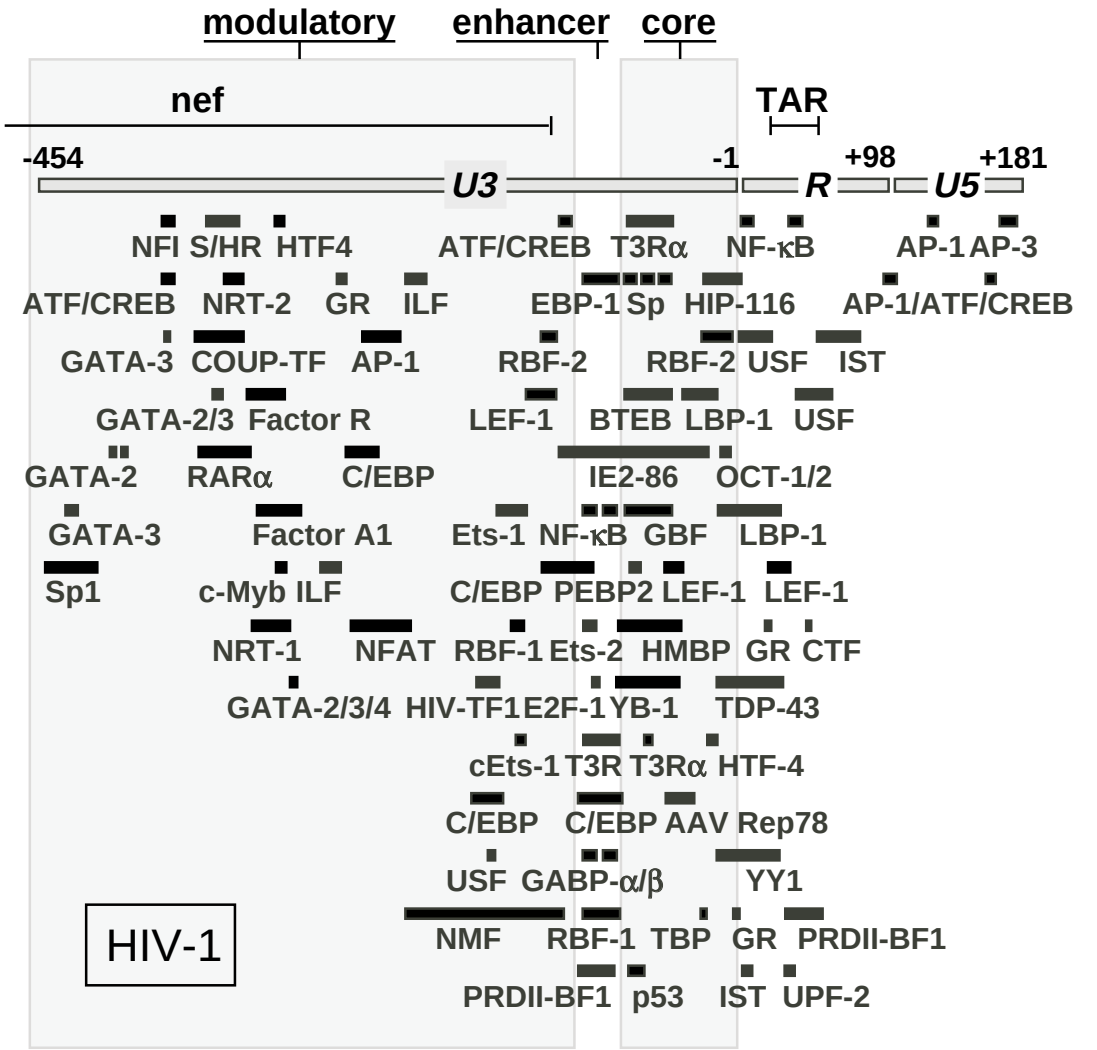


Figure 1. Detailed structure of the HIV-1 LTR. *Transcription factor binding sites identified within the HIV-1 LTR are shown with respect to the structural (U3, R, U5) and functional (modulatory, enhancer, core) divisions of the LTR. Transcription factor binding site locations were compiled from multiple reviews and publications (35-39).*

for the Sp family of transcription factors. The TATAA box binds TATAA binding protein (TBP) and a host of other proteins that comprise the RNA polymerase II transcription complex (35). Three tandem binding sites for Sp factors are positioned immediately 5′ of the TATAA box (40). The enhancer element, which is directly upstream of the core promoter and the Sp binding sites, is defined primarily by the presence of two copies of the 10 bp binding site for NF-κB and related factors (41). The modulatory region, which is comprised of sequences upstream of the NF-κB sites, contains binding sites for numerous factors, including CCAAT/enhancer binding protein (C/EBP) factors (42,43), activating transcription factor/cyclic AMP response element binding (ATF/CREB) factors (44,45), lymphocyte enhancer factor (LEF-1), nuclear factor of activated T cells (NF-AT) (46), and *ets*. Early investigations regarding the modulatory region suggested that factors binding in this segment of the LTR served to modulate the activity supported by the core and enhancer regions. Indeed, early experiments, in which deletion of upstream LTR sequences (5′ of nt -167) resulted in a 2-3 fold increase in LTR activity, led to naming this region the negative regulatory element (NRE) (47). Subsequent studies have shown this region to be rich in *cis*-acting binding sites, supporting both repression and activation of LTR activity (39). In summary, the plethora of transcription binding sites within the HIV-1 LTR provide numerous opportunities for cellular and viral transcription factors to interact and regulate LTR activity within the context of specific cell types, cell cycle regulation, cellular differentiation, and cellular activation. Furthermore, the activity of viral regulatory proteins, specifically Tat and Vpr (discussed below), add an additional layer of complexity to the regulation of LTR activity during the course of viral replication and HIV-1-associated disease.

A complete understanding of the structural and functional properties of the HIV-1 LTR requires analysis in the context of disease processes associated with HIV-1 infection. Toward that end, the following sections focus on LTR regulation within the context of specific aspects of retrovirology and pathogenesis, including (i) HIV-1 reservoirs and T cell activation, (ii) HIV-1 infection of monocytes and macrophages, (iii) the impact of chromatin on *in vivo* viral gene expression, (iv) HIV-1-associated CNS disease, (v) consequences of HIV-1 sequence variation, and (vi) activation of HIV-1 gene expression by the viral proteins Tat and Vpr.

1. LTR activity, T cell activation, and HIV-1 reservoirs

In 1995, investigations into the combined activity of nucleoside and non-nucleoside reverse transcriptase (RT) inhibitors, along with the newly-developed protease inhibitors which were also effective against HIV-1, suggested a new form of therapy for individuals infected with HIV-1. This new treatment regimen, referred to as highly active antiretroviral therapy (HAART), represented a breakthrough because of its ability to dramatically decrease levels of circulating virus (48), often to below the limits of detection, and to delay disease progression. However, subsequent analyses of patients undergoing HAART indicated that infectious virus could still be recovered and total eradication was unlikely because of the presence of reservoirs of latently-infected cells within the peripheral circulation and the immune system (49), and anatomical sanctuaries, such as the CNS, where the efficacy of drug treatments is lessened by constraints on drug penetration (50).

Investigations of HIV-1 reservoirs have focused on three cell types: macrophages, dendritic cells (specifically follicular dendritic cells or FDCs), and T lymphocytes. Macrophages, long identified as cells that support HIV-1 infection at levels lower than those of T lymphocytes, have been implicated as long-lived cells that produce virus following HAART (48) and an SIV reservoir after CD4-positive cell depletion subsequent to infection with a highly pathogenic strain of SIV (51). FDCs, which store large quantities of virus within lymphoid tissue, retain considerable numbers of virions even after extended triple drug therapy (52). Finally, investigations have revealed that the latently infected, non-activated CD4-positive T lymphocyte may be a predominant reservoir for HIV-1 in patients undergoing HAART (49,53,54). In non-compliant patients or patients undergoing "interrupted" therapy, HIV-1 may reemerge from the T cell reservoir by activation of the proviral genome. Because of the central role of the LTR in regulation of viral expression, efforts to understand the cellular events necessary to reactivate the latent HIV-1 genome in T cells must focus on regulation of LTR activity.

Studies of LTR function within the context of T cell activation often converge on the protein kinase C (PKC) signal transduction pathway and activation of NF-κB. Numerous investigations, beginning in

1987 (55), have linked members of the NF-κB transcription factor family to inducible LTR regulation in the T cell and other cell types through the interaction with the tandemly repeated, 10 bp binding sites of the enhancer region. Recent studies using the Jurkat T cell line have indicated that NF-κB can, in a manner independent of Tat, promote not only transcriptional initiation, but also elongation of the nascent transcriptional complex, to levels comparable to those of Tat (56). Removal of the NF-κB binding sites severely curtails basal (57) as well as Tat *trans*-activated LTR activity (57,58). NF-κB-induced LTR activity can be enhanced by the interaction of the LTR and HIV-1 nucleocapsid (NC) protein (59). Tat appears to be linked to NF-κB activation through a kinase that accelerates the degradation of IκB, a protein that regulates NF-κB activity by binding NF-κB and preventing its translocation from the cytoplasm to the nucleus (58). Binding of the cell surface receptor OX40 by its ligand gp34 results in induction of HIV-1 replication (60), which is dependent on the presence of the HIV-1 LTR NF-κB sites. OX40 is expressed on activated CD4-positive T cells, suggesting that this mechanism could be used to induce HIV-1 expression from latently infected T cell reservoirs. Changes that occur in cell cycle regulation during HIV-1 reactivation may also impact LTR activity and subsequent viral expression. E2F-1, a cellular transcription factor that stimulates S-phase gene expression, represses LTR activity in lymphocytes through the NF-κB p50 subunit and the LTR NF-κB binding sites (61). E2F-1 has also been shown to have similar activity in a wide range of cell types, including glial cells (62).

Concurrent infection by HIV-1 and other opportunistic agents may also affect HIV-1 expression from latently infected cells serving as viral reservoirs. Infection by human cytomegalovirus (HCMV), an opportunistic pathogen found frequently in AIDS patients, results in the expression of several immediate early (IE) genes, including IE86, IE72, and IE55, that are able to *trans*-activate the LTR through sequences in the modulatory region (-174 to -163) (63). In contrast, the UL44 gene product impedes the ability of Tat and the HCMV IE1/IE2 gene products to *trans*-activate the LTR (64), suggesting complex interactions between HIV-1 and HCMV. LTR activity has also been shown to be down-regulated by the latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus (KSHV), or human herpesvirus 8 (HHV-8) (65), indicating possible links between the pathogenesis of HIV-1 and Kaposi's sarcoma. Infection by hepatitis B virus (HBV), which is also commonly diagnosed in HIV-1-infected patients, results in up-regulation of LTR activity and viral replication through the HBV X protein, which synergistically activates the LTR with Tat and T cell mitogenic stimulation through the NF-κB and Sp binding sites (66).

2. Regulation of LTR activity in the infected monocyte/macrophage

Cells of the monocyte/macrophage lineage have critical importance in the progression of HIV-1 infection and disease. Monocytes and macrophages are the hosts of viruses of the CCR5-utilizing R5 phenotype, which is thought to be the predominating phenotype of viruses transmitted via a mucosal route. Differentiation of monocytes results in the production of non-dividing macrophages, which can serve as a reservoir of HIV-1 and a source of virus upon reactivation. Infected monocytes are also postulated to be one vehicle in which HIV-1 can be transmitted across the blood-brain barrier into the brain. Microglial cells, which are CNS-resident phagocytic cells of monocyte/macrophage lineage, likely harbor HIV-1 in the brain. However, the principal, productively infected host for HIV-1 in the brain may be the perivascular macrophage, as has been demonstrated in SIV-infected macaque brains shortly after infection, in animals with SIV encephalitis (SIVE), and during terminal AIDS (67).

Studies of HIV-1 LTR activity in monocyte cell lines and primary human macrophages have revealed the critical importance of HIV-1 LTR binding sites for C/EBP factors (43,68-70). C/EBP factors comprise a family of transcription factors of the b-ZIP superfamily. As with most b-ZIP transcription factors, C/EBP factors contain a basic region important in DNA binding as well as a leucine zipper structure, which mediates the protein-protein interactions necessary for homo- and heterodimerization. Although C/EBP proteins are expressed in a variety of tissues including hepatocytes and adipocytes, their expression in hematologic cells is primarily limited to the myeloid lineage. However, expression of different C/EBP family members is not uniform during the course of myeloid development; individual C/EBP factors are up- and down-regulated during differentiation. For example, during the progression of differentiation and macrophage maturation, C/EBPα, a transcriptional activator, is down-regulated while C/EBPβ (NF-IL-6) and C/EBPδ are up-regulated (71).

Multiple C/EBP sites have been localized within the HIV-1 LTR (42). One site (site I) is located immediately 5′ to the promoter-distal NF-κB site and immediately downstream of a binding site for factors of the activating transcription factor/cyclic AMP response element binding (ATF/CREB) family (45). Another is located further upstream (site II). Ross and coworkers have demonstrated that C/EBP factors that bind to site I interact with factors that bind the adjacent ATF/CREB site, adding an additional level of complexity to LTR activity in cells of the monocyte/macrophage lineage (72). Mutual interactions between C/EBP factors and ATF/CREB factors result in differential occupancy of each site, dependent on the ability of naturally occurring site variants to recruit members of each transcription factor family to their respective sites. Compartmentalization of sequence variation at C/EBP sites I and II and subsequent alterations in LTR regulation (73) may also play a role in the development of HIV-1-associated CNS disease, as discussed below. The resulting combination of differentiation-dependent C/EBP factor expression, sequence variation in both *cis*-acting sequences, and the number of functionally diverse factors available from each transcription factor family to occupy both sites, provide a wide latitude of regulatory mechanisms that may serve to regulate LTR activity within cells of monocyte/macrophage lineage.

Other cellular transcription factors may also play important roles in the regulation of LTR activity in HIV-1-infected monocytes and macrophages. For example, members of the Sp transcription factor family, which modulate LTR activity through three sites in the core region (40), include Sp1, Sp2, Sp3, and Sp4. Studies of the activities of these proteins have demonstrated that Sp1 and Sp4 activate the HIV-1 LTR, while over-expression of truncated Sp3 results in repression of LTR activity under certain experimental conditions (74). Recent investigations have revealed that Sp factor expression is dependent on monocytic differentiation (75) and that the Sp1:Sp3 ratio increases in a more mature monocytic cell line compared to less differentiated cells (McAllister and Wigdahl, unpublished observations). These results suggest that Sp-dependent LTR activity may be altered during the course of monocytic differentiation by changes in the Sp activator:repressor ratio or by differential covalent modification of Sp1 and Sp3.

3. In vivo occupancy of the LTR by nucleosomes

Studies of eukaryotic transcription and, in particular, HIV-1 LTR structure/function relationships have often been conducted using *in vitro* assays of LTR function and DNA-protein interactions: transient expression assays, *in vitro* transcription assays, *in vitro* footprinting, and electrophoretic mobility shift assays. However, following integration into the host genome, the proviral DNA must be expressed within the context of the organizational structure of the host cell DNA. *In vivo*, eukaryotic and proviral DNA is organized by histones and other proteins to form chromatin (76), which serves two purposes. First, it facilitates the storage of nuclear DNA in a highly compact form. Second, it regulates gene expression by restricting the access of transcriptional machinery to host and proviral genes and regulatory regions. The complex three-dimensional DNA-protein structure of chromatin limits associations between regulatory sequences and host transcriptional machinery, and inhibits conformational changes in the DNA necessary for transcriptional initiation and elongation (77-80).

The role of chromatin in the regulation of HIV-1 LTR activity and viral gene expression begins with the integration step of the replicative cycle. While HIV-1 integration is not sequence specific, studies have suggested that preferential integration takes place at or near locations with recognized sequence elements or specific DNA structures (81-83). Futhermore, the site of insertion influences both basal and Tat *trans*-activated HIV-1 LTR activity (84,85). The specific association of HIV-1 integrase (IN) with Ini1, a human homolog of a protein component of the yeast SWI/SNF complex, may also indicate a relationship between the site of integration and HIV-1 expression (86). Ini1 participates in a mammalian SWI/SNF complex, which has been shown in yeast to facilitate ATP-dependent chromatin remodeling and transcriptional activation (87). The association between HIV-1 IN and chromatin remodeling proteins suggests that HIV-1 integration may be directed toward regions of chromatin undergoing or disposed to active transcription.

Mapping the assembly of DNA-nucleosome complexes into chromatin can be accomplished using techniques that rely on digestion of the DNA within the chromatin. Differential sensitivity to endonuclease cleavage indicates the pattern of nucleosome loading; nuclease hypersensitive sites are thought to

represent regions of nucleosome-free DNA with roles in the regulation of gene expression (88). Multiple hypersensitive sites have been mapped along the HIV-1 proviral genome in chronically HIV-1-infected cell types representative of CD4-positive T lymphocytes and cells of monocyte/macrophage origin (89). Results of these studies demonstrated the presence of two DNase I-hypersensitive (HS) sites within the 5′ LTR: HS2 at nt 223-325 and HS3 at nt 390-449 (with respect to the 5′ end of the LTR) (Fig. 2). While HS2 and HS3 are still present in cells activated by phorbol esters or tumor necrosis factor alpha (TNF-α), the degree of hypersensitivity in the intervening sequence between HS3 and HS4 is increased (89). This same pattern of hypersensitivity, which is absent in quiescent monocytic cells, is present under basal conditions in chronically infected cells of T cell origin (89). Similar observations were made using micrococcal nuclease to digest nucleosome-free proviral LTR DNA (90). The conclusions drawn from these studies indicate, under basal conditions, the presence of two nucleosomes (nuc-0, nt 40-200; nuc-1, nt 465-610) on the 5′ LTR separated by a nucleosome-free region of approximately 265 nt (90) (Fig. 2). Under conditions that promote transcriptional activation, the presence of nuc-1, located immediately downstream of the transcriptional start site at the U3-R border, is disrupted, facilitating or permitting transcription (initiation and elongation) and association of transcription factors with binding sites in this region of the LTR (80). Supporting evidence for the latter was presented in studies in which mutations in three AP-1 binding sites and the AP-3-like motif (which binds the T cell-specific factor NF-AT), located within or in close proximity to the nuc-1 site, resulted in considerable reductions in both virus replication and LTR activity (91). The apparent importance of binding sites within this region of the LTR suggests that the disruption of nuc-1 is highly relevant to the support of productive HIV-1 replication.

Chromatin remodeling in the vicinity of the LTR can be achieved by a number of mechanisms. As indicated above, the association of nuc-1 can be disrupted by cellular activation using TPA or TNF-α (89). Consistent with the involvement of members of the NF-κB transcription factor family in T cell activation, studies have demonstrated that p65, in conjunction with Sp1 and factors that bind to upstream sites proximal to the NF-κB sites (LEF-1, Ets-1, and TFE-3), synergistically activates LTR-directed transcription on chromatin-assembled templates, but not on non-chromatin DNA (92). Interestingly, these transcription factors can gain access to their respective binding sites even in the presence of nucleosomes, suggesting that ternary complexes of transcription factors, DNA, and nucleosomes can form without prerequisite remodeling (93).

Remodeling can also be mediated by histone post-translational modification. Reversible acetyla-

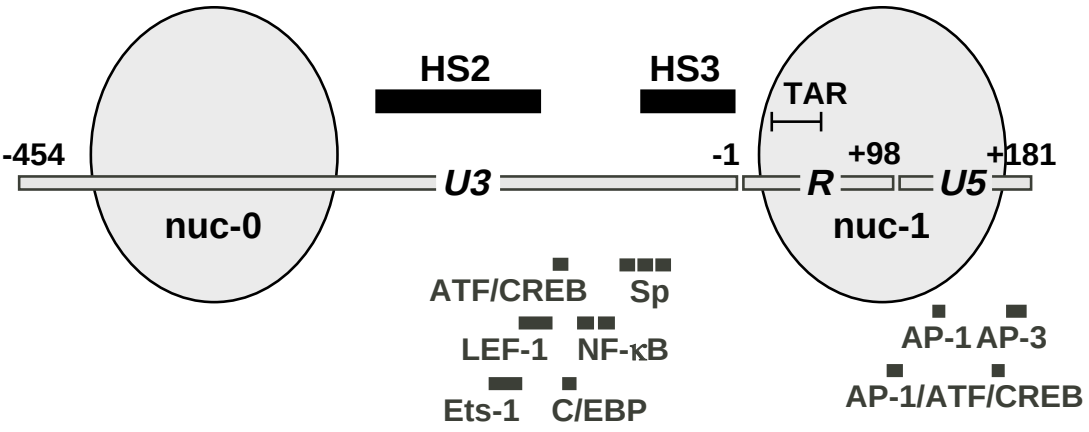


***Figure 2. Nucleosomes populate the HIV-1 LTR* in vivo.** *Positions of DNase I hypersensitive regions (HS2 and HS3) and locations of nucleosomes (nuc-0 and nuc-1) along the HIV-1 LTR are depicted. The locations of binding sites for transcription factors implicated in chromatin-dependent viral gene expression are also shown.*

tion of histone N-terminal tails has been recognized for its impact on gene expression from chromatin. The degree of acetylation, which disrupts DNA-histone interactions and increases DNA access, is controlled by a balance between the activities of nuclear histone acetyltransferases (HATs) and histone deacetylases. Numerous proteins involved in transcriptional regulation have been shown to also possess intrinsic acetylase or deacetylase activity, implying a close relationship between histone acetylation and chromatin regulation of gene expression. HAT activity increases LTR activity *in vitro* when the LTR has been pre-assembled into a nucleosomal template (94). With respect to Tat *trans*-activation, studies of Tat function have demonstrated that Tat associates with p300, P/CAF (95), and CREB-binding protein (CBP) (96), all of which have HAT activity. Using *trans*-dominant mutants of these proteins, experiments have demonstrated that P/CAF activity was necessary for Tat *trans*-activation of an integrated LTR (95). However, HAT-mediated chromatin remodeling and subsequent Tat *trans*-activation of the integrated LTR does not take place in the absence of the NF-κB and Sp1 sites (97).

4. Regulation of the HIV-1 LTR in the CNS

HIV-1 replication in cells within the CNS is a necessary component of the emergence of HIV-1-associated neuropathogenesis and disease. A multitude of studies have shown that cells that serve as hosts for HIV-1 within the CNS are identified primarily as cells of monocyte/macrophage lineage, including brain microglial cells. However, other cell populations can also support HIV-1 replication, albeit at levels below those found in cells of monocytic origin. Nevertheless, their cellular dysfunction and production of neurotoxic viral proteins likely contributes to the progression of CNS disease. In all of these cell types, HIV-1 expression and replication is regulated, in part, by the activity of the LTR (98).

Several investigations have shown that the LTR can direct gene expression within the CNS in a manner specific to both sequence and cell type. In studies in which LTR-β-galactosidase reporter expression was examined in transgenic mice, LTRs from brain-derived viruses were preferentially active over peripheral blood-derived LTRs in cells of the CNS (99). Developmental state may also impact LTR expression and subsequent viral replication, as shown by studies in which transgenic, brain-derived LTRs (JR-CSF and JR-FL) were differentially expressed during different stages of embryonic development (100). *In vivo* footprinting using transgenic LTR-reporter mice also revealed a DNA-protein interaction specific to the CNS-derived JR-CSF LTR and brain stem tissue (101), suggesting the activity of a transcription factor that is both brain- and LTR-specific. Consistent with these studies was the demonstration that sequence variations in LTRs isolated from HIV-1-infected brains were also found in previously documented, brain-derived LTRs (JR-CSF and JR-FL) and were distinct from a blood-derived LTR (IIIB) (102). HIV-1 LTR sequence compartmentalization has also been demonstrated. In comparisons of LTR sequences from infected cells in the blood, lung, and lymph nodes of HIV-1-infected patients, LTRs derived from the dorsal root ganglion and spinal cord were distinct from those isolated from the peripheral immune system and lung (103) (see consequences of HIV-1 sequence variation below).

The LTR may have a role in the progression of HIV-1-associated CNS disease and the appearance of HIVD beyond regulation of viral gene expression from integrated proviral DNA. Production of unintegrated proviral DNA within the host cell (including macrophages) is a characteristic shared by lentiviruses. Circular molecules of proviral DNA containing either one or two copies of the LTR are produced as non-integrating by-products following reverse transcription of the HIV-1 genome. Because these molecules do not integrate, they accumulate in the cell along with unintegrated, linear molecules of proviral DNA. The amount of circular DNA found in an infected cell may be correlated with the rate of viral replication, since, *in vitro*, infected monocyte/macrophage cells accumulate circular DNA at levels lower that those in productively infected T cells (104). Levels of unintegrated circular (and linear) DNA have been associated with HIV-1 encephalitis (105) and the appearance of multinuclear giant cells (a hallmark of HIV-1 CNS infection) and dementia (106). These studies have implicated unintegrated DNA as a marker for HIV-1-associated CNS disease, paralleling the link between the presence of unintegrated viral DNA in the blood and the severity of immunologic disease (107). Furthermore, unintegrated DNA in infected macrophages, microglia and T cells may serve as templates for the production of viral RNA and, subsequently, viral proteins implicated in neuropathogenesis, including gp120, Nef, and Tat (108). A low level of virus production from unintegrated proviral DNA in infected cells may contribute to the viral load in the brain, as has been demonstrated *in vitro* in HeLa cells (109).

In those unintegrated structures, expression of viral proteins as well as viral progeny would be regulated by interactions between cellular and/or viral proteins and the LTRs.

Studies have also been directed specifically toward understanding LTR structure/function relationships within cells of the CNS that support HIV-1 infection. Although cells of monocyte/macrophage/microglial origin have been identified as the primary host of HIV-1 within the CNS, other cells, including astrocytes, neurons, brain microvascular endothelial cells, and oligodendrocytes, may also be infected. Understanding LTR regulation specific to these cell types may facilitate a greater understanding of the contributions each cell type makes to the progression of HIV-1-associated disease.

Cells of monocyte/macrophage lineage have been shown to be the principal hosts of HIV-1 infection within the CNS (15). Extensive investigations have focused on two aspects of LTR function in these cell types pertinent to the regulation of viral gene expression in the CNS: (i) regulation of LTR activity through the GC-rich Sp binding sites, and (ii) *cis*-acting sequences that bind members of the C/EBP transcription factor family. The GC-rich region of the LTR immediately 3′ of the NF-κB sites binds factors of the Sp transcription factor family. The Sp family of transcription factors represent a group of phosphoproteins containing three highly homologous zinc fingers that facilitate DNA binding (110,111). Sp factors are generally ubiquitously expressed, suggesting that they support transcription in a wide variety of cell types. However, this group of transcription factors has recently been shown to be important in regulation of differentiation and cell cycle-specific processes, and, in particular, in the regulation of monocyte- and/or myeloid-specific gene expression (75,112-114).

McAllister and colleagues have shown a preferential utilization of Sp factors in T lymphocytes over cells of monocytic origin. In experiments to examine the importance of the most promoter-distal Sp binding site (site III), a low affinity binding site was substituted for the high affinity site III normally found in the LAI LTR. Replication of an LAI molecular clone with this substitution was markedly decreased in Jurkat T cells, but was reduced very little in U-937 monocytic cells (115). Furthermore, transient expression analyses indicated that the deficit in LTR activity in Jurkat cells caused by the site III substitution extended to both Tat- and Vpr-mediated activation of the LTR (115). These results suggest a decreased dependence on Sp factors for LTR activity in cells of monocytic origin.

Studies have also focused on the role of C/EBP factors in the control of LTR activity in the brain. As stated above, C/EBP factors are necessary for the maintenance of LTR activity and viral replication in monocytic cells (43,68,69). Ross and colleagues examined a large number of LTR sequences derived from HIV-1-infected brains and compared them to LTRs from the peripheral blood for sequence differences in C/EBP site I (promoter-proximal) and site II (promoter-distal) (73). The results indicated that brain-derived LTRs commonly possess a specific site I, the sequence of which results in enhanced binding of C/EBP factors. In contrast, there was no preference for this site in peripheral blood LTRs. Furthermore, site II was more highly conserved in the brain LTRs than in LTRs derived from peripheral blood. Transient transfection analyses using the brain-derived YU-2 LTR (116) also indicated that Tat *trans*-activation of the LTR was dependent on the highly conserved, strong-binding site II. The preferential appearance of C/EBP site sequences in LTRs isolated from the brain suggested that these sites play important roles in LTR regulation of HIV-1 gene expression during viral invasion and maintenance within the CNS.

The observations that LTR sequences are compartmentalized and may undergo selection upon entry or during infection of the CNS have been incorporated into two opposing hypotheses of CNS invasion and the development of HIV-1-associated CNS disease. HIV-1 invasion of the brain generally occurs early in the infection (15). However, while the progression of peripheral immune dysfunction and the appearance of CNS disease and HIVD are not necessarily linked temporally, HIVD does not generally appear before the onset of severe immunodeficiency (15). The delay in onset, often measured in years, may be a consequence of viral clearance or, at least, latency (117). The frequent post-mortem localization of HIV-1 to perivascular cells of monocyte/macrophage/microglial lineage suggests that reseeding of the brain late in disease by activated HIV-1-infected monocytes may initiate the final sequence of pathogenic events in the brain (117). The implication of this hypothesis is that viruses (and LTR sequences) found in the brain during HIV-1-associated CNS disease arise from cell populations in the bone marrow and evolve in the peripheral circulation. In this scenario, viral evolution (including the evolution of the LTR)

would be driven by selective pressures within cells of the peripheral immune system and would take place during replication in the T cell population or, perhaps, in the course of viral evasion of immune surveillance in infected cells of monocyte/macrophage lineage. The opposing hypothesis is that the initial seeding event marks the beginning of an independent course of viral evolution within the brain that may culminate in the onset of HIV-1 CNS disease (118). If the second hypothesis is correct, LTR evolution relative to the etiology of CNS disease would be driven not by the peripheral blood, but by influences within the brain, primarily in cells of the monocyte/macrophage lineage. Ultimately, both mechanisms may contribute to the overall evolution of the LTR and HIV-1-associated CNS disease.

While cells of monocyte/macrophage lineage are the principal hosts of HIV-1 in the CNS, astrocytes may also be important hosts for infection and contributors to CNS disease. The role of astrocytes in HIV-1-associated CNS disease (119) has been expanded considerably by numerous investigations that have shown that astrocytes are susceptible to HIV-1 infection *in vitro* and harbor HIV-1-specific nucleic acids and proteins *in vivo*, as shown by post-mortem analyses of brains from AIDS patients with encephalopathy or HIVD (15). While astrocytes support considerably lower levels of replication than do immune-derived cells, the astrocytic cell population may serve as a reservoir for HIV-1, seeding the CNS with virus following reactivation by cytokines associated with CNS infection. Additionally, disruption of normal cellular functions by HIV-1 infection may contribute to cytokine and chemokine dysregulation associated with HIV-1 CNS infection (15).

In astrocytes, the role of Tat in regulating LTR activity has been an area of particular focus. Early studies demonstrated that HIV-1 was capable of TAR-independent replication, but only in the presence of Tat and intact NF-κB binding sites (120,121). Subsequent investigations showed that TAR-independent LTR *trans*-activation in astrocytes did not require Tat RNA binding (122) but did require the activity of an astrocyte-specific factor associated with NF-κB (122). RNase protection assays performed with U-87 MG astrocytoma cell extracts suggested that TAR-independent Tat *trans*-activation functions at the level of transcriptional initiation, whereas TAR-dependent Tat activity involves enhancement of elongation (123). Transcription factor YB-1 may serve to enhance TAR-dependent LTR *trans*-activation by facilitating interactions between Tat and the TAR (124). NF-κB also participates in modulation of astrocytic gene expression by extracellular Tat, as shown in experiments in which application of extracellular Tat resulted in increased NF-κB binding to the LTR and induction of the PKC signal transduction pathway (125). The significance of TAR-independent Tat *trans*-activation beyond HIV-1 replication is that other cellular promoters in astrocytes (and neurons), including promoters for transforming growth factor beta-1 (TGF-β1) and TNF-α can also be up-regulated by Tat (126), possibly through the interaction between NF-κB p50 and Tat at promoters that possess NF-κB binding sites (127). However, despite the demonstration of two separate pathways for Tat *trans*-activation, HIV-1 replication in astrocytes is curtailed considerably compared to replication in lymphocytes or monocyte/macrophage cells (128).

Investigators have demonstrated other influences on HIV-1 LTR activity in astrocytes. Expression of the c-Ha-ras gene in astrocytes has been shown to up-regulate LTR activity and viral replication through the LTR enhancer, implicating the participation of the membrane signal transduction protein p21 ras in HIV-1 gene expression in astrocytes (129). Other studies have demonstrated that the HIV-1 accessory protein Vpr is capable of *trans*-activating the LTR in U-87 MG cells, and the activity of Vpr is dependent on the presence of Sp1 and the GC-rich sequences of the core region (nt -80 to -43) and is decreased by overexpression of p53 (130). As has been shown in neuronal cells, transcription factor YB-1 in U-87 MG cells activates LTR activity, which is inhibited by competitive binding of Sp1 to its binding sites (131). Other transcription factors shown to interact with the LTR in astrocytic cells include AP-1 (132) and members of the ATF/CREB transcription factor family (45).

The role of Nef during HIV-1 infection of astrocytes is also an area of continued study. Nef is expressed *in vivo* in HIV-1-infected astrocytes in adult and pediatric patients (15) and *in vitro* during the restricted infection of astrocytoma cells (133). Although Nef can have a pleiotropic effect on LTR activity and viral expression (134,135) as well as reduce CD4 cell surface presentation (136), Nef enhances viral expression in astrocytes (137). However, expression of Nef in human astrocytoma cells results in down-regulation of expression, activation of PKC isoforms and the concomitant inhibition of LTR activation

by phorbol esters (138), implying a negative influence of Nef under some circumstances. The exact role of Nef in astrocyte infection and HIV-1-associated CNS disease remains to be determined.

While neurons may only represent a small fraction of the cells infected within the brain (139,140), viral gene expression may nevertheless impact the progression of HIV-1-associated neurologic disease through mechanisms that include Tat neurotoxicity, deregulation of cytokine networks within the brain, and neuronal death or dysfunction (15). Neurons are capable of low level virus production after infection *in vitro*, which can be inhibited by neuronal differentiation (141,142). Extracellular Tat, which has been implicated as a neurotoxin involved in HIV-1 CNS disease (15), is also capable, in non-toxic concentrations, of *trans*-activating LTR activity and enhancing virus expression in human neuronal cells (143). As in immune cells, NF-κB appears to be a key mediator of LTR activity in neurons (144). In primary rat hippocampal and cerebral cortex neurons, the constitutive activity of NF-κB supports increased LTR activity, which is abolished by co-expression of an NF-κB inhibitor (145). The core region of the LTR, which contains three GC-rich binding sites for members of the Sp transcription factor family, is also involved in neuronal LTR regulation, as demonstrated using the SK-N-MC human neuronal cell line. In these *in vitro* experiments, YB-1 activation of the LTR through GC-rich sequences (nt -80 to -43) was decreased by co-expression of Sp1, apparently due to competitive binding between YB-1 and Sp1 (131).

Differences in the nuclear milieu specific to cell type and lineage may also modulate LTR activity in neurons. In studies in which distinct lineages of dividing, immature neuronal cell lines were used to assess neuronal LTR function, mutational analyses of the LTR resulted in the identification of LTR sequences (nt -255 to -166) involved in lineage-specific LTR activity (146), suggesting the activity of neuronal lineage- or differentiation-specific transcription factors. Dependence of LTR *cis*-acting site utilization on differentiation state was also demonstrated in transient expression analyses in which differentiation of embryonal carcinoma NTERA-2 cells to a neuronal phenotype by retinoic acid resulted in differential use of LTR sequences, especially those in the modulatory region (nt -112 to -453) (147).

Co-infection by other opportunistic agents within the CNS, particularly HCMV, may affect the course of HIV-1 replication and disease by altering the activity of the LTR in HIV-1-infected cells. In primary human astrocytes and the SK-N-MC human neuronal cell line, co-infection with HIV-1 and HCMV results in increased p24 (HIV-1 capsid or CA) production, paralleling increases in LTR activity in CMV-infected cells transfected with an LTR reporter (148). Similarly, CMV and human herpesvirus type 6 (HHV-6) stimulate both HIV-1 replication and basal LTR activity in human fetal astrocytes, in part through enhancement of transcription factor occupancy at AP-1 binding sites (nt -354 to -316) located within the LTR modulatory region (149). However, other investigations have suggested that the relationship between HIV-1 and HCMV is more complex. Using human cell lines of neuronal, astrocytoma, and glioblastoma origin, *in vitro* experiments demonstrated that co-infection with HCMV and HIV-1 can have no effect on HIV-1 gene expression, or result in either repression or transient activation of HIV-1, dependent on the permissivity for CMV expression and the degree of HIV-1 expression (150).

Extracellular, soluble signaling mediators within the CNS, including cytokines, chemokines, neurotransmitters, growth factors, and other molecules, may also impact LTR function and thereby viral gene expression. Nerve growth factor (NGF), a neurotrophic factor that induces neuronal differentiation as well as survival, recruits factors other than NF-κB to the enhancer region of the LTR and activates the LTR in a Ras/Raf-dependent manner (151). Similarly, NGF enhances HIV-1 replication in human neuroblastoma cells and, to a greater extent, in a glial cell line with an immature phenotype (152), suggesting that NGF may contribute toward the progression of CNS disease in pediatric cases of HIV-1 infection. In human cortical neuronal HCN-1A cells, HIV-1 infection is enhanced three-fold by the application of NGF or fibroblast growth factor (FGF) (153). Nitric oxide (NO), a cellular metabolite linked to HIV-1-associated neurotoxicity (15), inhibits LTR induction and HIV-1 replication in a human astrocytoma cell line through inhibition of NF-κB activity (154). TNF-α, interleukin-1 beta (IL-1β), and IL-6, which are cytokines involved in complex intercellular interactions that support the progression of HIV-1 CNS disease, have been shown to activate the LTR to varying degrees in cell lines of human neuroglial origin (155). Experiments that demonstrated enhancement of LTR activity and HIV-1 replication in human fetal brain perivascular microglia by the endogenous opiate β-endorphin (156) and

induction of LTR activity in human neuroblastoma cells by morphine (157) suggest that opiate drug abuse and pain management using morphine in HIV-1-infected patients may affect HIV-1 replication in the CNS.

5. Consequences of HIV-1 LTR sequence variation

It has long been known that the HIV-1 genotype and resultant phenotype are important variables with respect to viral replication, and that these variables change over the course of HIV-1 infection to impact disease progression (158-161). HIV-1 genotype variants are generated over the course of disease due to the low fidelity of reverse transcriptase, a lack of proofreading by the viral polymerase, rates of viral production, and *in vivo* selection pressures (162). During progressive HIV-1 infection, swarms of virus progeny containing variant genomic sequences are continually being produced (163). Many of these progeny viruses have broadened viral tropism and increased cytopathic capacity (158,160,161,164,165). The overall viral population will be dominated by those strains that are most fit at the time (166). Recombination may also contribute to the diversification of HIV-1, which may occur between highly divergent strains or related members within the viral variants that evolve within an infected individual during the course of disease. In support of this hypothesis, studies have indicated that dual infection of HIV-1-positive individuals by multiple HIV-1 subtypes and subsequent inter-subtype recombination plays an important role in the establishment of HIV-1 sequence diversity (166,167).

Goodenow and colleagues demonstrated that these swarms of HIV-1 variants cannot be described in simple molecular terms, but should rather be considered viral quasispecies (168). HIV-1 quasispecies contain sequence changes throughout the viral genome, including the LTR. The phenomenon of quasispecies development compounds the complexity of regulated viral expression by the LTR, since *cis*-acting transcription factor binding sites within the LTR may be altered functionally by sequence changes that arise during quasispecies evolution. These sequence changes may subsequenly result in functionally-altered LTRs that modulate viral gene expression and give rise to viral quasispecies with enhanced or decreased replication capabilities

Quasispecies that evolve geographically within the HIV-1-infected population are classified according to sequence identity into subtypes or clades. LTRs from numerous clades have been studied and characterized by both sequence and function (Fig. 3). Gao and coworkers have identified subtype-specific sequence motifs in the viral LTR that distinguish clade E viruses from all others including clade A (162). A hybrid between clade A and E, produced by clade recombination which most likely occurred in Central Africa, has been designated CRF01_AE. Gao and coworkers analyzed the regulatory regions of the CRF01_AE LTR, looking for alterations in known regulatory binding sites. The CRF01_AE LTR contains a single NF-κB site (AGGACTTCC) that differs from consensus sequence (GGGRNNYYCC), because it contains a transition at the 5′ end and a single deletion (162). LTRs from most other HIV-1 clades contain two or three NF-κB sites, suggesting that NF-κB-induced LTR activity may differ between the CRF01_AE LTR and other LTRs. CRF01_AE contains a GABP binding site, which replaces the upstream NF-κB site found in other clades (169,170). CRF01_AE has also been shown to have a TBP binding site with the sequence TAAAA, whereas all other clades and SIV_{cpz} have a TATAA sequence. There were also changes in the TAR element, which contains a stable RNA stem-loop structure 5′ end essential for Tat-mediated *trans*-activation (162). CRF01_AE has a two-nucleotide bulge in the TAR where most others have a three-nucleotide bulge. Changes in the TAR structure could affect Tat-mediated transcription as well as the initial steps in the reverse transcription process (162).

Rodenburg and coworkers have discussed the effect that NF-κB has on transient expression and viral promoter activity. They performed transient expression analyses with Clade C LTRs, which contain three NF-κB sites (Fig. 3), and demonstrated increased activation as compared to LTRs with only one or two NF-κB sites. These results have led to the hypothesis that the presence of three NF-κB binding sites may have spurred the more rapid spread of subtype C viruses compared to any other subtypes (171). These results have also been demonstrated by other investigators (172-174), including Naghavi and coworkers, who demonstrated that subtype C viruses exhibited increased p24 (CA) levels and thus higher replication than viruses of other clades (172). A correlation between the copy number of *cis*-acting transcription factor binding sites and increased LTR function is consistent with studies that have shown that duplications in the HIV-1 Sp binding sites result in increased LTR activity and greater replication rates (175-177).

Naghavi and coworkers also examined LTRs from viruses of different clades to document changes in other sites within the LTR (Fig. 3). The TCF-1α site was the most variable site; only 1 LTR out of 29 had a consensus B site. The negative regulatory element (NRE) had only 3 out of 29 which were consensus B. The distal Sp1 (site III) exhibited the greatest variation of the three Sp1 sites. The Sp1 sites, NF-κB sites, TATA, and TAR were all relatively conserved. NF-κB III was consensus B in 26 out of 29 LTRs and NF-κB II was consensus B in 27 out of 29 LTRs. Clades C, D and CRF01_AE all contained subtype-specific sequences in the NRE. All subtype C LTRs contained three NF-κB sites (172). Thus, genetic diversity of LTR may result in HIV-1 subtypes with different replicative properties. Sequence variability in the LTR may contribute to differences in the virulence of different HIV-1 subtypes.

As discussed above, studies have shown that LTR sequence variation plays an important role in HIV-1 replication and, specifically, in CNS tropism (99). More recently, we demonstrated that specific HIV-1 LTR C/EBP configurations preferentially encountered in the brain exhibited enhanced LTR-specific activity (73). An enhanced affinity C/EBP site I (6G configuration, the nucleotide at position 6 of the C/EBP site varies from consensus T to a G) was commonly found in brain-derived LTRs, but was infrequently encountered in peripheral blood-derived LTRs, as demonstrated by analyses of variations at each nucleotide position within site I (Fig. 4A). A differential conservation was also observed at C/EBP site II. Analyses of overall conservation of each site showed that C/EBP site II was highly conserved in LTRs derived from brain and less conserved in LTRs derived from peripheral blood (Fig. 4B). Transient

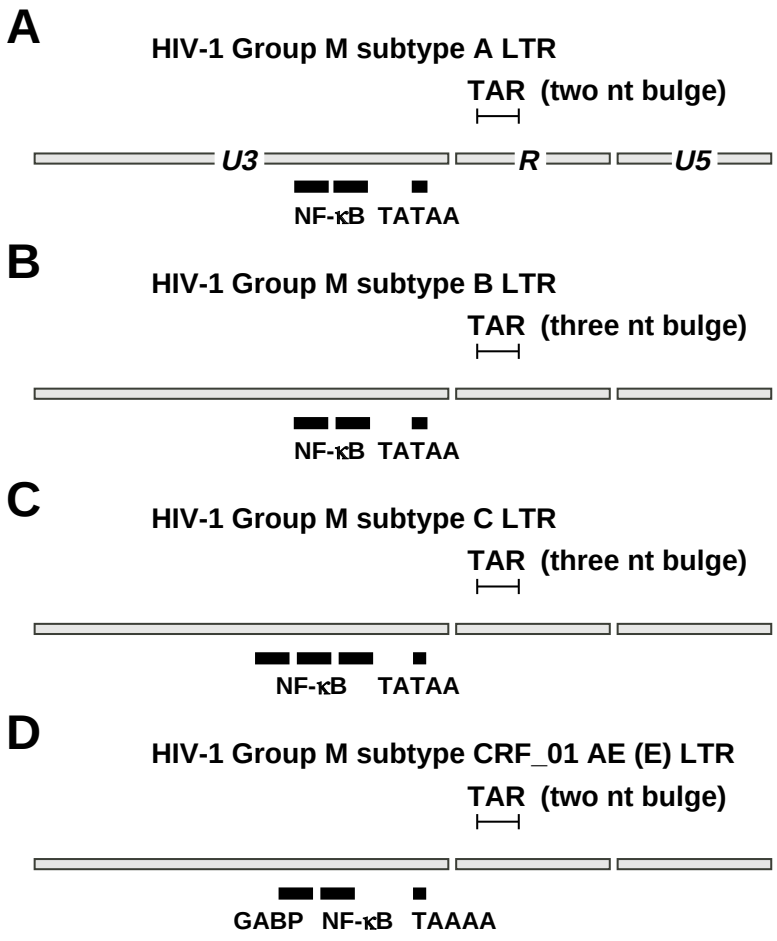


Figure 3. Comparative structures of HIV-1 Group M subtype LTRs. *Schematic comparison of HIV-1 LTRs from Group M subtypes A, B, C, and E (CRF01_AE).*

expression analyses indicated that inclusion of the 6G C/EBP site I resulted in increases of basal and IL-6-induced LTR activity. Overall, these studies demonstrated that brain-derived LTRs contain two relatively high affinity C/EBP binding sites, which suggests that these sites may play a role in LTR-directed transcription especially in CNS disease (73).

A study of multiple LTR sequences derived from different tissue types of a single HIV-1-infected individual indicates that LTRs derived from specific tissues may be classified as phylogenetically distinct variants, suggesting that LTRs may evolve in a tissue compartmentalized fashion (103). Phylogenetic analyses of HIV-1 LTR quasispecies from lymph node, spleen, lung, dorsal root ganglion, spinal cord, and peripheral blood have indicated that the LTR sequence variants cluster according to their tissue origin.

A

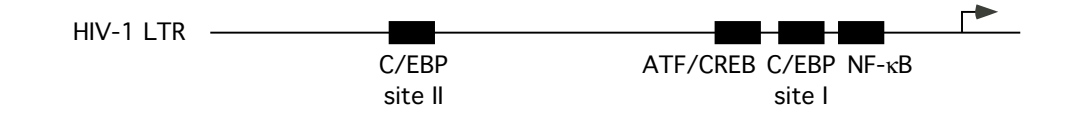
Mean nonconsensus frequency per nucleotide position

ATF/CREB	C	T	G	A	C	A	T	C	G
Peripheral Blood	*	2	1	10	3	*	21	6	*
Brain	7	*	*	*	9	*	13	1	1

NF-κB	G	G	G	A	C	-	-	-	T	T	T	C	C
Peripheral Blood	3	1	*	*	1	*	*	*	*	*	*	*	2
Brain	3	4	*	1	*	1	1	1	*	*	1	*	*

C/EBP site II	A	T	T	T	C	A	T	C	A
Peripheral Blood	3	5	4	17	2	26	6	*	*
Brain	8	*	6	3	*	3	5	1	*

C/EBP site I	A	G	C	T	T	T	C	T	A	C	A	A	-
Peripheral Blood	7	5	11	3	4	6	3	2	4	2	2	*	3
Brain	*	3	23	3	2	43	5	*	*	*	1	*	*



B

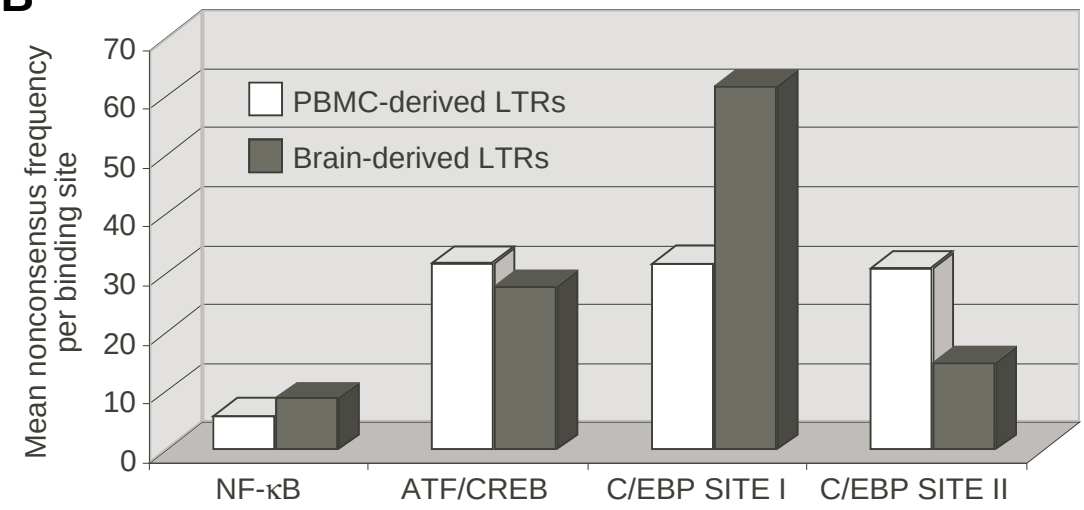


Figure 4. Brain-derived LTRs have unique sequence configurations at C/EBP sites I and II compared to peripheral blood-derived LTRs. *The frequency of a non-clade B nucleotide at each position was calculated for LTRs from each individual. The mean non-consensus frequency (NCF) was calculated from these values for each nucleotide position for the population of individuals. (**A**)* Cis*-acting binding sites for the following transcription factors were included in the analyses: NF-κB, ATF/CREB, C/EBP site I, and C/EBP site II. The bar below position 6 of C/EBP site I identifies the position of the common guanine substitution frequently found in brain-derived LTRs. Asterisks (*) indicate mean nonconsensus frequencies of less than 1%. (**B**) NCFs for each position were used to calculate a mean NCF for each site. This figure was adapted with permission from Ross* et al.*, 2001 (73).*

Spinal cord and dorsal root ganglion variants retained prototypic NF-κB binding sites, but exhibited seven sequence alterations within the TAR sequence which significantly reduced binding. Deviation from the consensus sequence was more likely within transcription factor binding sites and, therefore, consistent with evolution of LTR variants most fit for survival in specific cell types. Low level infection of the CNS may have occurred early in the course of HIV-1 disease followed by tissue-specific adaptation of the LTR, leading to HIV-1 variants optimally responsive to transcription factors predominately in the cells of nervous system tissue. Thus, LTR sequence variation may indeed play a role in adaptation of the virus to the CNS as well as to other tissues. In an earlier study, Ait-Khaled and coworkers conducted similar phylogenetic analyses and demonstrated a distinct polarization between HIV-1 variants from peripheral blood and lymph nodes in patients with intact lymph nodes (178).

Evidence supporting a role for the LTR in cell-type specific viral replication was provided by the finding that viral replication in primary monocytes, but not primary T cells, requires the presence of intact C/EBP sites within the viral LTR (69). These results demonstrated that *cis*-acting elements within the LTR might exhibit different patterns of utilization in T cells and monocytes. This evidence is consistent with the hypothesis that cell-type specific transcription factors or factor combinations can be utilized by properly adapted viral promoter structures to guide HIV-1 viral replication within specific cell lineages.

However, direct examination of LTR sequences with respect to disease progression has failed to reveal any significant association. A number of studies have compared LTR sequences from HIV-1-positive long-term nonprogressor and rapid progressor cohorts (175,179,180). In each case, no simple correlation between specific LTR sequence variants and the rapidity of disease onset was identified. Moreover, in two of the three studies the transcriptional activity of variant LTRs was examined by transient expression analysis in both cell lines and peripheral blood mononuclear cells (PBMCs) (179,180). These studies indicated no significant relationship between promoter strength and disease progression. However, there were two individual nonprogressors who exhibited potentially defective LTRs. Sequences from one patient in the Zhang cohort (one of eight) exhibited G to A hypermutations throughout the promoter structure, suggesting a defective 5′ LTR. Similarly, a long-term nonprogressor from the Rousseau study (one of four) exhibited an array of insertions and deletions across the LTR, again suggesting a potentially defective LTR structure. These observations suggest that defective LTR structures may predict long-term nonprogression. However, due to the variety of structural, enzymatic, and regulatory activities required for efficient HIV-1 replication, it is not surprising that the majority of nonprogressor cases are correlated with other non-LTR sequence variations. Each of these studies demonstrated a high level of conservation among core and enhancer LTR regions, while more frequent variation was observed in the modulatory region, particularly outside the *nef* gene.

There is evidence that other regions of the LTR are important in LTR basal activity, replication, and the production of progeny virus. Deletion of the TATA box greatly reduces basal LTR activity and greatly impairs viral replication in tissue culture cells (181-183). The three Sp binding sites that comprise the remaining sequences in the core region are also very important to HIV-1 basal replication. Mutation of the three sites diminishes both basal promoter activity and viral replication (although not in all cell types) (40,184-186). Millhouse and coworkers demonstrated that specific Sp site III sequence variations present in the brain-derived HIV-1 variant, YU-2, fail to interact with members of the Sp transcription factor family. This result, combined with the observation that the ratio of Sp1:Sp3 binding to site III is increased with the degree of monocytic differentation (75), suggests that HIV-1 replication within cells of monocyte/macrophage lineage within the brain may be impacted by changes in Sp factor expression that accompany monocytic differentiation as well as alterations of the functional interactions between Sp factors and the NF-κB proximal, G/C-rich Sp binding site. Sp site sequence variation and altered Sp factor recruitment may also impact the ability of HIV-1 Vpr to *trans*-activate the LTR (as discussed below) (187). In addition, recent studies have implied a functional convergence between C/EBP site variants preferentially found in HIV-1-infected brains (73) and Vpr, an HIV-1 accessory protein that interacts directly with C/EBP sites I and II in the HIV-1 LTR (188). Other studies have shown that recruitment of C/EBP factors to site I can be affected by sequence variation and differential factor recruitment to the adjacent ATF/CREB site (72). These latter results indicate that analyses of the impact of binding site sequence variation must also take into account the effect of factors recruited to adjacent sequences.

In summary, an analysis of currently available studies has indicated that the contribution of LTR sequence variation to HIV-1 disease pathogenesis is complex. Significant evidence exists which indicates that LTR variation may alter promoter activity in different cell populations and tissue types. The disease progression studies discussed suggest that sequence variation may influence the rapidity of pathogenesis in certain circumstances. LTR variation may play a role in tissue-specific disease, such as HIVD, or in the maintenance of viral reservoirs in particular cell populations during retroviral therapy.

6. Activation of the HIV-1 LTR by virus-encoded proteins

Using only the host cell transcriptional machinery, the LTR is capable of supporting relatively low levels of basal transcription. However, basal transcription from the LTR is generally insufficient to support productive replication. To overcome this limitation, expression of the HIV-1 genome results in the production of Tat, which is capable of inducing LTR activity and greatly increasing viral expression.

Tat (Fig. 5A) acts through a unique RNA regulatory segment of the HIV-1 LTR designated the TAR (*trans*-activation-responsive) element (37). Deletion analysis of the nucleotides between +19 to +43 in the LTR R region demonstrated that the TAR element was necessary for Tat activity. It was found that TAR functions as an RNA element forming a highly stable stem-loop structure consisting of 26 nucleotide

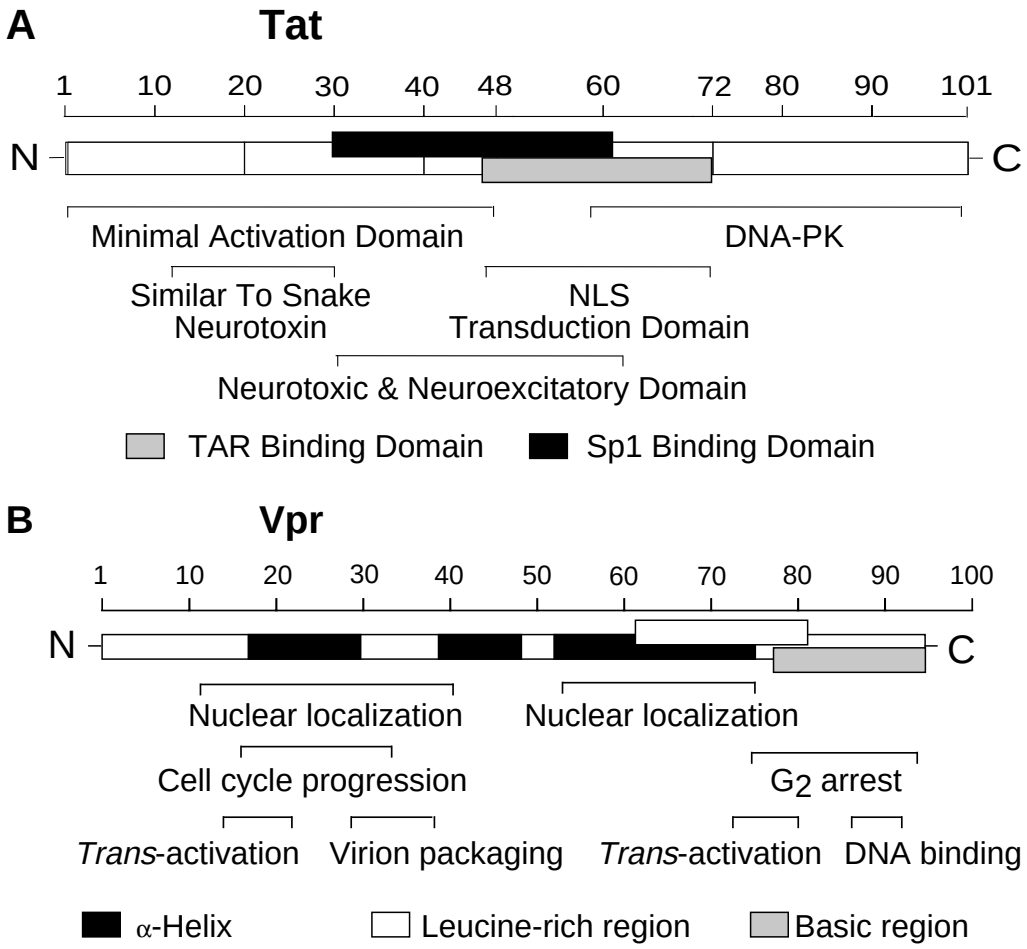


Figure 5. Activation of the LTR by Tat and Vpr. (A) *Locations of functional domains of Tat. The gray box depicts the TAR binding domain and the black box depicts the Sp1 binding domain.* ***(B)*** *Functional domains of Vpr. Vpr has many functions as depicted here. The domain important for* trans*-activation of the LTR lies between amino acids 12-23.*

pairs and a nucleotide bulge that interfaces with the viral transcription activator protein Tat (189). Mutations that disrupt TAR base pairing eliminate Tat-driven LTR transcription (190). It is widely held that the Tat-TAR interaction serves to tether Tat and allow it to interact with the basal transcriptional machinery. Because generation of the RNA stem loop structure requires transcription of the first 44 nucleotides of the nascent mRNA (191), Tat-mediated transcriptional activation was thought to be generated at a post-initiation step, possibly through interactions with host cell elongation factors. Intense investigations by numerous groups have delineated such a mechanism. In the absence of Tat protein, relatively low levels of viral gene expression can be driven by the proviral LTR or by an LTR fused to a reporter gene (192).

Additionally, studies have demonstrated that Tat also facilitates enhanced transcriptional initiation (191,193,194). In this role, Tat activation may be facilitated through a protein-protein interaction with the cellular transcription factor Sp1 (193). This hypothesis is supported by the finding that mutation of the *cis*-acting elements which recruit Sp factors to the HIV-1 LTR generated a reduction in Tat-mediated LTR activity (184). In addition, synthetic promoters have been demonstrated to be Tat-inducible in a Sp1-dependent manner (195). Hence, Sp factors binding to the GC rich region of the HIV-1 LTR may facilitate Tat recruitment proximal to the basal transcriptional machinery. Sp1-dependent Tat *trans*-activation may also be linked to cell cycle events, as shown by experiments in which the LTR was activated by Tat in the G_1 phase in a TAR- and Sp1-dependent manner (196). Conversely, in the G_2 phase, Tat was shown to activate LTR activity in a TAR- and Sp1-independent manner (196).

There are other roles for Tat beyond activation of the HIV-1 LTR. Tat is capable of modulating the expression of cellular genes, such as the gene for manganese-dependent superoxide dismutase (197). During the development and progression of HIV-1-associated neuropathogenesis and dementia, Tat can function as a neurotoxin (15). In addition, Tat is involved in cell cycle progression through interactions with cyclin-T, specifically with the cdk9-cyclin T complex. Numerous reviews of HIV-1 Tat provide more extensive details regarding the functions of Tat and the mechanisms that support those functions (198,199).

Another viral protein capable of augmenting HIV-1 LTR activity is Vpr (Fig. 5B). Vpr has a diverse range of functions, including regulation of HIV-1 pre-integration complex nuclear import and arrest of cell cycle progression in proliferating cells (200). However, the first function attributed to Vpr was the ability to enhance activity of the HIV-1 LTR as well as several heterologous promoters (201). Vpr-mediated LTR activation has been demonstrated in a variety of cellular contexts with induction levels generally on the order of 4- to 14-fold (compared to up to 100-fold activation by Tat) (130,187,202-204). Some studies have suggested that Vpr-mediated transcriptional activation is an indirect effect of its ability to arrest the cell cycle (200,205). Other evidence indicates the involvement of more direct interactions between Vpr and elements of cellular transcription.

Vpr may mediate LTR activation by interacting with transcription factors bound to the LTR or by direct protein-DNA interactions. Vpr interacts with DNA by way of a positively charged C-terminal domain (206). In addition, Vpr has also been shown to form direct protein-protein interactions with TFIIB (207). Due to TFIIB's role in the basal transcription initiation complex, Vpr may facilitate promoter activation through recruitment of this complex. Deletion analyses of the HIV-1 LTR have demonstrated that Vpr-mediated LTR activation is mediated through the GC box array (187). This *cis*-acting structure is responsible for recruiting Sp factors to the HIV-1 LTR, a process critical to viral replication. Interestingly, Wang and coworkers demonstrated that Vpr associated with Sp1 in the context of the GC box array, but not with either Sp1 or the *cis*-acting element alone. These results indicated that a ternary complex might be required to facilitate the interaction. Moreover, shorter DNA sequences capable of binding single Sp factors were also inadequate to support Vpr binding even in the presence of Sp1. However, additional studies indicate that under optimized reaction conditions, Vpr is capable of binding a variety of DNA and RNA sequences (206). Vpr binding appeared to be dependent on the length of DNA or RNA elements examined, although Zhang and coworkers were unable to identify a particular binding element that exhibited enhanced Vpr binding. Despite the lack of a specific *cis*-acting element responsible for Vpr recruitment, the results reported in their study indicated that direct DNA-protein interactions may play a role in Vpr-mediated LTR activation. Using electrophoretic mobility shift analyses, we have

demonstrated direct interactions between a Vpr-GST fusion protein derived from the 89.6 dual tropic strain of HIV-1 and C/EBP sites I and II (188). This interaction is particularly strong using a C/EBP site I variant (3T) that has a relatively low affinity for C/EBP factors. The implication of this observation is that Vpr may modulate HIV-1 expression in cells of monocytic lineage through interactions with C/EBP factors and their *cis*-acting sites.

Because Vpr is present in the viral particle in relatively large quantities (approximately 1200 copies per virion) (201), it is believed that Vpr may function in the up-regulation of viral gene expression in newly infected cells before the appearance of Tat. Interestingly, Vpr function in the presence of Tat has yet to be fully delineated. Cotransfection of Tat- and Vpr-expressing constructs mediated an additive activation of the HIV-1 LTR in U-87 MG cells (204), while the direct addition of Tat protein to HEK 293 cells transfected with increasing amounts of Vpr expression construct demonstrated a dose-dependent inhibition of Tat-mediated LTR activity (203). Moreover, the recent segregation of Tat-mediated LTR activation into cell cycle-dependent components (196) implies that Vpr may influence Tat activity through its ability to mediate cell cycle arrest. Additional studies that better elucidate the function of Vpr in the presence of Tat may be critical to fully understanding Vpr function relevant to HIV-1 gene regulation.

B. Role of the HIV-2 LTR in disease pathogenesis

Like the HIV-1 LTR, the HIV-2 LTR is divided structurally into the U3, R, and U5 regions (Fig. 6A). The 5′ LTR of HIV-2 contains a TAR element located downstream of the transcriptional initiation site in the R region (208,209). Unlike the HIV-1 TAR element, which contains a single stem-loop, the HIV-2 TAR element consists of two characteristic stem-loop structures, both of which participate in optimal Tat response (208,209). While the HIV-1 TAR element is able to respond to HIV-1 and HIV-2 Tat equally well, the HIV-2 TAR element responds to HIV-2 Tat somewhat more efficiently than to HIV-1 Tat (208-212). HIV-2 gene expression is also modulated by sequence elements downstream of the transcriptional initiation site, corresponding to the U5 region of the LTR and sequences further downstream (213).

The HIV-2 LTR is responsive to T cell activation signals, but the regulatory elements required for the response differ from those of HIV-1 (214). The HIV-2 LTR is less responsive than the HIV-1 LTR to T cell activation signals and the transcriptional enhancer determines the phenotypic response of the LTR to PHA and PMA (212). The HIV-2 enhancer binds NF-κB and AP-3 (212). The HIV-1 transcriptional enhancer contains two conserved NF-κB sites, while the corresponding region in the HIV-2 LTR contains only one conserved NF-κB binding site (212). The entire HIV-2 LTR has a 40% sequence similarity with that of the HIV-1 LTR (23), whereas their TATA boxes, Sp1 sites, and transcriptional enhancers have 50% sequence similarity. The TATA box and three Sp1 sites in HIV-2 are located within 80 nucleotides of the site of transcription initiation and have functional similarities to those described for the HIV-1 LTR (212). Both HIV-1 and HIV-2 contain transcriptional enhancer regions upstream from the Sp1 sites (212). The HIV-2 LTR lacks the NFAT binding site and the negative regulatory elements present upstream from the promoter region in the HIV-1 LTR (212). In comparing and contrasting the HIV-1 and HIV-2 LTRs, it is tempting to speculate that differences between the LTRs may account for at least some of the dramatic differences in pathogenesis between these two viruses. However, studies to date have not directly addressed this hypothesis.

While the T cell activation response element of HIV-1 is a single direct repeat of an NF-κB binding site, the response element of HIV-2 is more complex (214,215). Substitution mutation analysis of the HIV-2 LTR has revealed it to be composed of additional *cis*-acting, synergistic sub-elements (216,217). In addition to the single NF-κB site, the HIV-2 LTR contains at least four other *cis*-acting enhancer elements: two purine rich sequences (PuB1 and PuB2), a peri-ets (pets) sequence, and a monocyte-specific peri-κB sequence (216,218-220). The two purine box elements are recognized by Elf-1, an Ets-related transcription factor protein expressed in T cells (217). Sequence analyses of these transcription factor elements indicated that they are highly conserved *in vivo* (28) and across most HIV-2 isolates. Functional studies demonstrated that antigen-mediated transcriptional activation of the HIV-2 enhancer required the presence of PuB1 and PuB2, the pets site, and the NF-κB site (220). HIV-2 transcriptional

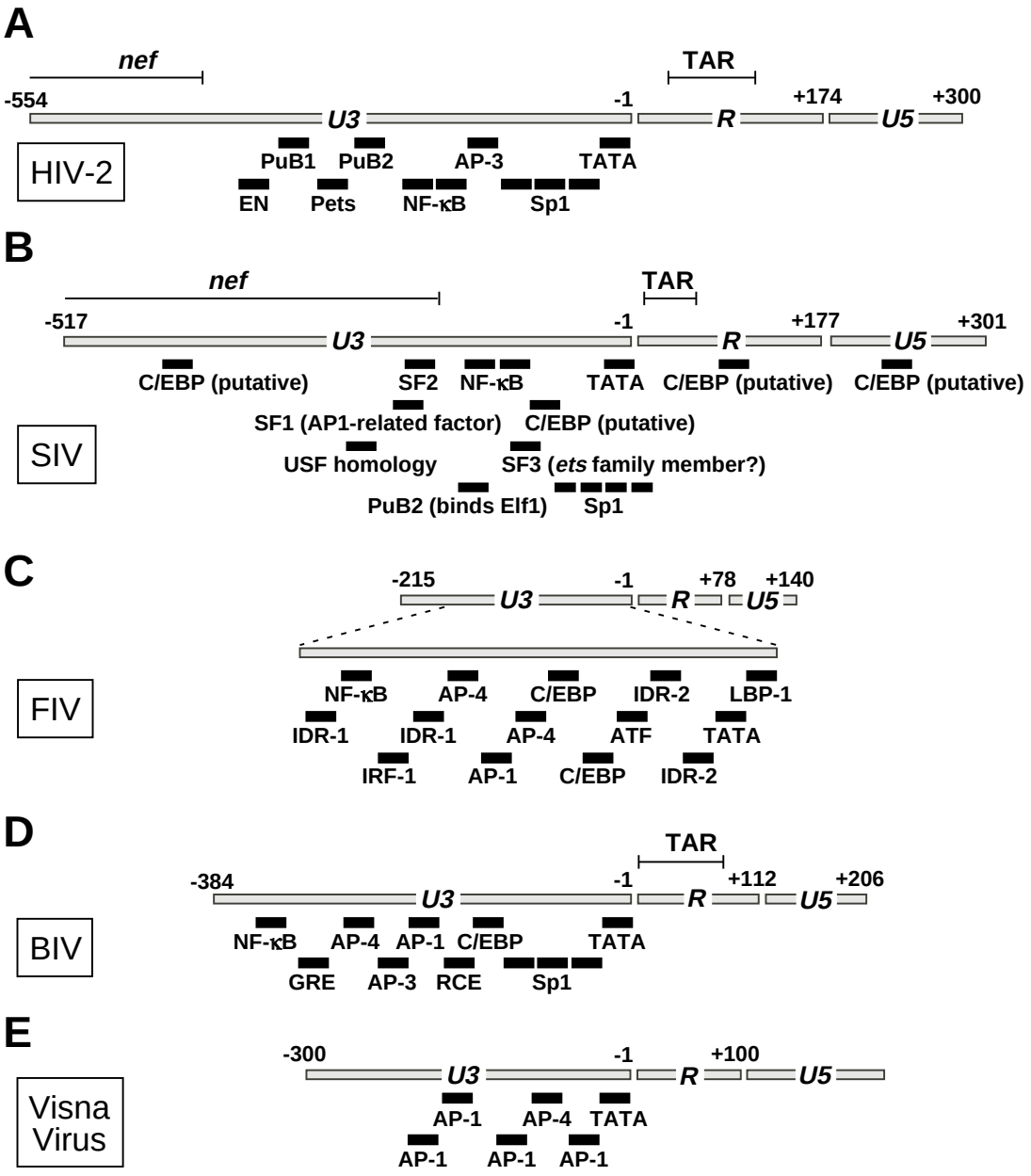


Figure 6. Comparative structures of other lentiviral LTRs. *(**A**) The U3, R, and U5 regions of the HIV-2 LTR and the locations of transcription factor binding sites that have been identified, including the NF-κB binding site, three Sp1 binding sites, and the TATA box, are depicted (28,220). (**B**) Locations of known or putative transcription factor binding sites are depicted to scale relative to the SIV LTR and its structural and functional subdivisions. The promoter distal NF-κB site is not present in all strains of SIV. The locations of putative binding sites for members of the C/EBP transcription factor family were determined using both sequence analyses and DNA-protein binding studies (Hogan and Wigdahl, unpublished observations). (**C**) Depiction of the FIV LTR U3, R, and U5 regions and positions of the IRF-1, AP-1, AP-4, C/EBP, ATF, imperfect direct repeat (IDR), and TATA binding sites (242,245,273). (**D**) A representation of regulatory elements within the BIV LTR (247,248). (**E**) Illustration of the visna virus LTR with locations of the TATA box, and AP-1 and AP-4 binding sites indicated (251,257,274).*

regulation by factors in T cells was distinguished from HIV-1 transcriptional regulation because HIV-2 was more inducible by agents that mimic T-cell antigen receptor signaling (215). Induction of HIV-2 transcription may proceed through a mechanism that utilizes the same regulatory proteins in activated T cells that increase IL-2 transcription (220). The HIV-2 transcriptional control elements (PuB1, pets, PuB2, and a NF-κB site) play roles in supporting basal HIV-2 transcription as well as inducible transcription after treatment with stimulatory agents, including phorbol ester, plant lectin, or anti-CD3 antibodies (220). These elements are also necessary for the antigen-MHC-mediated induction of HIV-2 gene expression in 2B4.11antigen-specific T-cell hybridoma cells (220).

Unlike the HIV-1 and SIV, *cis*-acting sequences for members of the C/EBP transcription family have not been identified in the HIV-2 LTR. The apparent lack of C/EBP sites within the HIV-2 LTR suggests that transcriptional mechanisms other than those involving C/EBP factors are used to support HIV-2 expression in monocytes and macrophages. However, the HIV-2 LTR contains other novel elements that function in a cell type-dependent manner (221). These elements, which are located upstream of the enhancer-promoter region, are more active in T cells compared to monocytic cells and are modulated by the activity of HIV-2 Tat (221).

Because HIV-1 and HIV-2 are co-endemic in certain geographic areas, the consequences of concurrent infections with HIV-1 and HIV-2 have been investigated. There are instances of dual infection, despite the lower risk of HIV-1 infection to HIV-2-infected individuals (222). HIV-2 inhibits HIV-1 replication and the mechanism of inhibition was found to be suppression of the HIV-1 LTR by HIV-2 (222). The inhibitory effect does not appear to be associated with Tat-2, but rather with differences in the TAR elements of each LTR (222). These results suggest both a molecular mechanism for HIV-2 interference with HIV-1 replication and a potential molecular approach to therapy (222,223). HIV-2 has been shown *in vitro* to efficiently inhibit HIV-1 super-infection, whereas HIV-1 only partially interferes with HIV-2 super-infection (224). HIV-2 provirus inhibited HIV-1 replication when the provirus was transfected into cells. HIV-2 provirus *trans*-activates the homologous HIV-2 LTR efficiently, but *trans*-activates the heterologous HIV-1 LTR poorly (222). The inhibitory effect of HIV-2 appears to discriminate between the HIV-1 and HIV-2 LTRs based on differences in the TAR element (222).

C. Participation of the SIV LTR in disease pathogenesis

Although the SIV LTR is generally similar to the HIV-1 LTR, there are some important organizational and functional distinctions (Fig. 6B). In 1990, Renjifo and coworkers demonstrated that there were *cis*-acting elements in the U3 region of the SIV LTR similar to those in HIV-1. They analyzed the sequence of $SIV_{mac}142$ and identified three motifs related to Sp1 consensus binding sites (-52 to -80) and a single copy of the NF-κB consensus sequence (-96 to -105). Through deletion analyses and utilization of chloramphenicol acetyltransferase (CAT) transient expression assays, they demonstrated that the NF-κB site and sequences -162 and -114 contain *cis*-acting regulatory elements that support viral transcription in Rat-1 cells. They reported evidence that the U3 region of the SIV LTR can act as a positive regulator of basal expression in Rat-1 and Jurkat cells (225). Anderson and coworkers demonstrated that LTR sequences from -225 to +18 were sufficient to maintain full transcriptional activity of both $SIV_{mac}239$ and $SIV_{mac}251$ in Hut-78 and U-937 cell lines. DNase footprinting analyses demonstrated an NF-κB footprint as well as an additional footprint at -52 to -38 utilizing Hut-78 and U-937 nuclear extracts (226).

The presence of a second NF-κB site in some SIV LTRs may have pathogenic consequences. The $SIV_{smmPBj1.9}$ strain, which contains a 22 bp duplication in the LTR that generates a second NF-κB site, replicates to high titers in unstimulated cells and is acutely pathogenic in pig-tailed macaques (227). In contrast, the SIV_{agm3mc} strain, which is apparently non-pathogenic in pig-tailed macaques and African green monkeys, contains an enhancer with a single NF-κB site and replicates in PBMCs only after mitogenic stimulation. Studies using hybrid viruses constructed from these two strains have shown that viral replication in unstimulated cells is dependent on the presence of the $SIV_{smmPBj1.9}$ U3 region (228), suggesting that the LTR may contribute to the more pathogenic phenotype.

Winandy and coworkers identified a factor that binds to a *cis* element overlapping the NF-κB region of the SIV LTR (229). The factor, which was named simian factor 3 (SF3), was shown to play a role in

the basal regulation of the SIV LTR, whereas under conditions of induction, NF-κB would act in this region. SF3 was also shown to possibly bind to an element in the -162 to -114 region. Two other factors were also shown to bind in this region. One, which was designated simian factor 1 (SF1), is a ubiquitous basal factor, and the other, simian factor 2 (SF2), is a T cell-predominant, phorbol myristate acetate-inducible factor (229) (Fig. 6B). In 1996, Murakami and coworkers demonstrated that the SF1 element, which spans nucleotides -135 to -131, interacts with AP-1-related factors (230). These factors were shown by UV cross-linking experiments to be part of the Jun/Fos family of proteins. They characterized a specific SF1 binding factor in the nuclear extracts of T cell and monocytic cell lines. SF1 was found in all cell types and was unaffected by treatment with PHA, PMA, or calcium (230).

Because the *nef* reading frame overlaps about 70% of the U3 region of the 3′ SIV LTR, considerations of LTR structure and activity must also include Nef. SIV viruses require an intact *nef* gene in order to induce disease (231). The Nef protein acts as an important virulence factor *in vivo* in monkeys. It has been demonstrated that the *nef* gene of SIV is necessary for efficient growth in H9 (T lymphocytes) cells (232). The molecular clone SIV_{mac}239-nef-deletion, which encodes a truncated *nef* gene, is non-pathogenic and, in H9 cells, replicates with delayed kinetics (232). Nef may help accelerate the progression of the disease in monkeys, since infection of rhesus monkeys by a *nef*-deleted SIV virus results in low viral titers and a slower rate of AIDS development (231). To study the importance of upstream LTR sequences independent of the *nef* gene, Ilyinskii and coworkers utilized three mutants of the upstream region of the LTR: two mutants with conservative and non-conservative sequence changes that did not alter the *nef* coding sequence and an upstream deletion mutant (233). All three viruses constructed with these mutated LTRs replicated with similar kinetics or slightly delayed kinetics in macaque PMBCs and CEMx174 cells. When rhesus monkeys were infected with viruses containing the conservative or non-conservative LTRs, there was no decrease in virulence (233), indicating that the function of upstream LTR sequences is primarily to encode Nef and not the modulation of LTR activity, viral expression, or replication.

This same group demonstrated that SIV molecular clones without NF-κB or Sp1 binding elements were able to transcribe and replicate efficiently in PBMCs (234). However, these mutant viruses failed to replicate in cells of the monocyte/macrophage lineage. Thus, NF-κB and Sp1 elements are necessary for efficient viral replication of SIV in cells of the monocyte/macrophage lineage, but dispensable for efficient viral replication in lymphocytes. Ilyinskii and coworkers also reported the induction of simian AIDS by SIV lacking NF-κB and Sp1 binding elements (235). Mutant strains of SIV_{mac}239 with changes or deletions in the four Sp1 sites and NF-κB site were capable of replicating in lymphoid cells *in vitro* to a level similar to that of the parental strain. Rhesus monkeys were infected with mutant strains containing deletions or substitutions of Sp1 and NF-κB sites. All but one of the infected animals exhibited an early plasma antigen, maintained high virus burdens, and had significant changes in lymphoid tissues, and six died with AIDS within the first 60 days. In contrast, HIV-1 variants containing deletions or substitutions in all NF-κB and Sp1 elements were not competent for replication (235).

Upstream LTR sequences may support SIV transcription in the absence of the core enhancer promoter. Unlike the HIV-1 LTR, the SIV LTR contains an enhancer region just upstream of the NF-κB region that supports a significant level of transcription in the absence of the NF-κB and Sp1 sites (234). A purine-rich site (PuB2) in the 26 bp of sequences located just upstream of the NF-κB binding site in the SIV LTR binds specifically to the transcription factor Elf-1, a member of the *ets* proto-oncogene-encoded family (236). This regulatory element allows efficient viral replication in the absence of the entire core enhancer region. The HIV-2 LTR also contains purine-rich sites (PuB1 and PuB2) that are the targets of an *ets* oncogene, Elf-1. Both the SIV and HIV-2 LTRs differ from the HIV-1 LTR because they contain one NF-κB site, and binding sites for the *ets* oncogene family members, including PuB2, which binds Elf-1. In addition, C/EBP binding sites within the SIV LTR have been putatively identified (Hogan and Wigdahl, unpublished observations) (Fig. 6B). However, unlike the C/EBP sites in the HIV-1 LTR, the roles of the SIV sites in viral replication, particularly in cells of monocyte/macrophage lineage, have not yet been established.

D. Other lentiviral LTRs

While other animal lentiviruses, including feline immunodeficiency virus (FIV), bovine immunodeficiency virus (BIV) and visna virus, are not as widely studied as HIV-1 or SIV, they provide valuable insights into the molecular biology, cell biology, and disease processes associated with lentiviral infections. Like HIV-1, FIV is capable of infecting T lymphocytes and monocytes/macrophages, causing a fatal acquired immunodefiency syndrome-like disease in cats (237). Likewise, because of similarities between the neurologic consequences of HIV-1 infection and visna virus infection of sheep, this virus is considered the prototypical lentiviral model for retroviral-induced neurodegenerative disorders (238). Structure/function studies of LTRs from these viruses may also provide enlightenment with regard to HIV-1-associated disease.

1. Feline immunodeficiency virus (FIV)

Studies of the FIV LTR have highlighted the importance of several *cis*-acting sites within the LTR, including sites that bind AP-1 and ATF transcription factors (Fig. 6C). AP-1 and AP-4 binding sites, contained within a 31 bp segment of the LTR, are involved in sustaining basal LTR activity (239) but are apparently unnecessary for the maintenance of virus replication in concanavalin-A-stimulated feline PBMCs or in feline T cell lines (239,240). In experiments in which LTR sequences were progressively deleted, deletion to the AP-1 site (approximately 124 bp upstream of the site of transcriptional initiation) resulted in a 10- to 25-fold decrease in LTR activity (241). Further deletions through the ATF binding site (54 bp upstream) severely reduced LTR activity (241). These sites also confer LTR responsiveness to T cell signal transduction pathways, including those involving PKC and protein kinase A (242). In studies in which mutated LTRs were incorporated into FIV infectious molecular clones, deletion of the ATF site decreased viral replication in both feline lymphocytes and macrophages, while deletion of the AP-1 site had no effect on viral replication (243). A more extensive deletion, which included the AP-1, ATF, and C/EBP sites, also resulted in a severe reduction in replicative capacity (243). Sequences that encompass these three sites are also important for induction of LTR activity by the FIV *trans*-activator protein, which shares characteristics with Tat proteins found in other lentiviruses (244).

Like HIV-1, the FIV LTR is dependent on the activity of C/EBP transcription factors to support viral gene expression and replication. In viral replication studies, mutation of the FIV LTR C/EBP site resulted in decreased replication in both feline kidney and T-lymphoblastoid cells (245). Furthermore, the C/EBP site was shown to participate in inhibition of FIV LTR activity by pseudorabies virus ICP4 (245). Studies also suggest that factors that act through the C/EBP site may affect FIV LTR activity in a cell-specific manner (241,245).

2. Bovine immunodeficiency virus (BIV)

The BIV LTR (clone 127; 589 nucleotides in length) contains numerous *cis*-acting regulatory sequences. Initial sequence analyses indicated the presence of elements related to binding sites for Sp1 and NF-κB transcription factors (246) (Fig. 6D). Subsequent studies demonstrated the functionality of NF-κB, GRE, AP-4, AP-1, CAAT, and ATF/CREB binding sites; these sites activate the LTR during both basal and Tat *trans*-activated transcription (247). In contrast, binding sites for AP-4 and Sp1 had effects of LTR activity that varied with cell type and Tat *trans*-activation (247). The same group of investigators also identified sequences spanning the U5 and adjacent untranslated regions that repressed LTR activity (247). Like HIV-1, BIV Tat, which is required for BIV replication, acts through a TAR in the R region (nucleotides 1-31) to *trans*-activate the LTR (248). Cell type-dependent, TAR-independent *trans*-activation has been noted, albeit at lower levels than TAR-dependent *trans*-activation (247). However, unlike HIV-1 Tat, BIV Tat does not require the participation of cyclin T1 for TAR binding, but does require it for LTR *trans*-activation (249).

3. Visna virus

Visna virus (maedi-visna virus or MVV) infects cells of monocyte/macrophage lineage in sheep and induces the slow and progressive development of clinical abnormalities associated with pathogenesis primarily in the lungs and CNS (250). Like other lentiviruses, MVV expression is controlled by an LTR with *cis*-acting regulatory sequences (Fig. 6E) and is activated by MVV Tat (251). Within the LTR, six putative AP-1 sites and a single AP-4 site were initially identified and found to support basal transcription (251,252). The TATA-proximal AP-1 site was also shown to participate in Tat *trans*-activation (251),

which involves protein-protein interactions between Tat, c-fos, c-jun, and TATA binding protein (TBP) (253). The demonstration that cellular transcription factors c-fos and c-jun, which bind AP-1 sites, mediate LTR activation by Tat and phorbol esters in macrophages establishes a direct link between induction of MVV expression and macrophage maturation, which also relies on *fos*- and *jun*-regulated gene expression (254,255). Like HIV-1, MVV is subject to the appearance of genomic variants during the course of infection (256). Sequence variations that arise within the LTR may be determinants for cell tropism, as suggested by experiments in which viruses containing two different LTR variants grew equally well in macrophages but replicated at different rates in non-macrophage cell types (257). Unlike HIV-1, visna virus apparently does not require functional C/EBP sites for replication in cells of monocyte/macrophage lineage.

4. Equine infectious anemia virus (EIAV) and caprine arthritis/encephalitis virus (CAEV)

Studies of lentiviruses not associated with immunodeficiency have also provided highly relevant observations that provide links between LTR activity and disease progression. For example, the LTR of EIAV, infection of which produces anemia and wasting in horses, contains an enhancer region that appears to play an important role in the degree of virulence, tropism for host cells (macrophages, fibroblasts, and endothelial cells), and differential expression associated with monocyte differentiation (258-260). Surprisingly, compared to the relatively low level of sequence diversity in other regions of the LTR (~5%), the enhancer region is considerably hypervariable (~45%) (261), perhaps due to selective pressures associated with cell-specific utilization of transcription factor binding motifs contained within the LTR (259).

Like EIAV, replication of CAEV is also linked to monocytic differentiation. The cytokine gamma interferon (IFN-γ), which is released in response to viral infection by activated T cells and natural killer cells, results in monocytic differentiation as well as activation of the CAEV LTR and increased viral expression (262,263). Interestingly, IFN-γ treatment of U1 cells (a monocytic cell line chronically-infected with HIV-1) also results in induction of HIV-1 expression (264), suggesting a similar link between HIV-1 LTR activity and IFN-γ-induced differentiation.

IV. Concluding Remarks, Future Questions, and Speculative Comments

The LTR, like its eukaryotic promoter counterparts, is of central importance to the regulation of gene expression. The LTR serves as a convergence point for numerous transcription factors that, themselves, serve as end-point effectors in numerous signal transduction cascades and other regulatory activities initiated both inside and outside the host cell. The activities of these factors are integrated by the LTR to regulate viral gene expression, the production of progeny virus, and the spread of the infection. Additionally, the LTR regulates the expression of viral gene products, such as HIV-1 Tat, Vpr and gp120, that have a detrimental impact on cellular functions, host cell viability, and intercellular communication via cytokines and other soluble mediators.

The HIV-1 LTR is a particularly complex example of a eukaryotic promoter, which operates at several levels of regulation. The first level, basal transcription, is supported by interactions between the LTR, the host cell transcriptional machinery, and the widely expressed Sp1. At the second level is transcription activated by intra- and extra-cellular events that affect transcription factors that interact with the LTR. One such example is the activation of the LTR by activation of members of the NF-κB transcription family. In an exquisite example of exploitation, HIV-1 uses the PKC signal transduction pathway and the mechanism for NF-κB activation, which is an integral part of the regulation of immune cells, in the support of LTR regulation and viral gene expression in cells in which this pathway is most important. The cellular functions of other transcription factors, including C/EBP, NF-AT, and ATF/CREB, have been similarly recruited for the activation of LTR activity. The third level of regulation consists of viral proteins, including Tat and Vpr, that activate the LTR to support higher levels of viral gene expression. While the central function of Tat appears to be LTR *trans*-activation, the nature of Vpr's role as an LTR activator in the context of its effect on cell cycle progression and its role in nuclear import will require further study.

Further complicating the view of LTR function are several factors related to cell biology and virology. Studies have shown that LTR activity can be impacted by the progression of the host cell through the cell cycle and by cellular differentiation. Since HIV-1 is capable of infecting a range of cell types, including T lymphocytes, monocytes, macrophages, microglial cells, astrocytes, neurons, microvascular endothelial cells, and others, LTR function can be affected by transcription factors and signal transduction pathways specific to those cell types. Additionally, the generation of viral quasispecies with diverse LTR sequences can result in the production of viruses that have a spectrum of replicative capacities as a consequence of altered LTR function. One means by which LTR variants may be generated is evolution of the virus as it passes between T lymphocytes and cells of monocyte/macrophage lineage. Different selective pressures in these cell types may result in specific alterations in LTR sequence and function that may, in turn, affect the replicative capacity and pathogenic potential of viruses in the immune system and in the CNS.

The summation of these activities results in the expression of HIV-1 gene products and the production of progeny virus, which, ultimately, advances the progress of the infection and the disease processes associated with infection (Fig. 7A). In this way, LTR function has a direct impact on the pathogenesis and clinical characteristics associated with HIV-1 infection. Clearly, the study of HIV-1 LTR function is an important component of achieving a greater understanding of the pathogenesis of HIV-1 infection.

There are numerous pathogenic consequences of LTR function to be considered (Fig. 7B). For example, LTR function may facilitate viral survival during the initial infection and subsequent viremia. LTR variants with low activity that arise shortly after infection may allow populations of infected cells to evade immune surveillance during the early viremia and the subsequent immune response that marks the end of the acute phase and the beginning of the clinical latency phase of the disease (14,18). The effect of cell type, particularly cells of monocyte/macrophage lineage that support generally lower levels of HIV-1 expression than T cells in the peripheral blood, might also contribute to the preferential survival of HIV-1-infected cells during the early stages of disease. Viruses carrying LTRs with weak C/EBP sites, which are unable to support levels of LTR activity necessary for HIV-1 replication in monocytes, might evade clearance and, later in the disease, replicate to higher levels in T cells, which do not require functional C/EBP sites (43,68,69). Alternatively, the presence of a low affinity Sp site III in the LTRs of viral populations infecting T cells would result in considerable decreases in viral replication, again possibly facilitating evasion of immune clearance (115). The dissemination and evolution of these surviving viral populations later in the course of infection might also be facilitated by changes in the extracellular environment or changes in cellular location (e.g. peripheral blood to brain) that affect cellular differentiation, activation, and proliferation, and, subsequently, the ability of the LTR to support increased replication.

Sequence- and cell-specific LTR function may also impact the course of CNS disease associated with HIV-1 infection (117,118) (Fig. 7B). The general evolution of HIV-1 within the peripheral immune system is from a CCR5 phenotype early in disease to a CXCR4 phenotype in the terminal stage of AIDS. Consequently, viral populations that seed the CNS during the initial infection may differ phenotypically from viruses that enter the CNS during the development of AIDS. If the virus is seeded into the CNS early in disease and evolves independently in that compartment throughout the course of the infection, the virus (and LTR) may evolve under different selective pressures (primarily in perivascular macrophages and brain microglial cells or possibly in astrocytes or neurons) compared to viruses that evolve in the peripheral blood (primarily in T cells but also in cells of monocyte/macrophage lineage) and subsequently enter the CNS later in the disease. Our studies have demonstrated that variants of HIV-1 LTR C/EBP sites I and II appear with divergent frequency in the brain and peripheral blood (in LTRs primarily derived from T cells) (73). The preferential appearance of high affinity C/EBP sites in LTRs derived from HIV-1-infected brain tissue suggests that viruses with LTRs that have higher basal activity and are more responsive to IL-6 within the brain are positively selected over viruses with less responsive C/EBP sites. These viral populations may have evolved in the brain over the extended course of infection from viruses that entered the CNS early in the acute phase (118). Alternatively, there may be a course of evolution within the peripheral immune system (perhaps beginning in the bone marrow and later in the peripheral

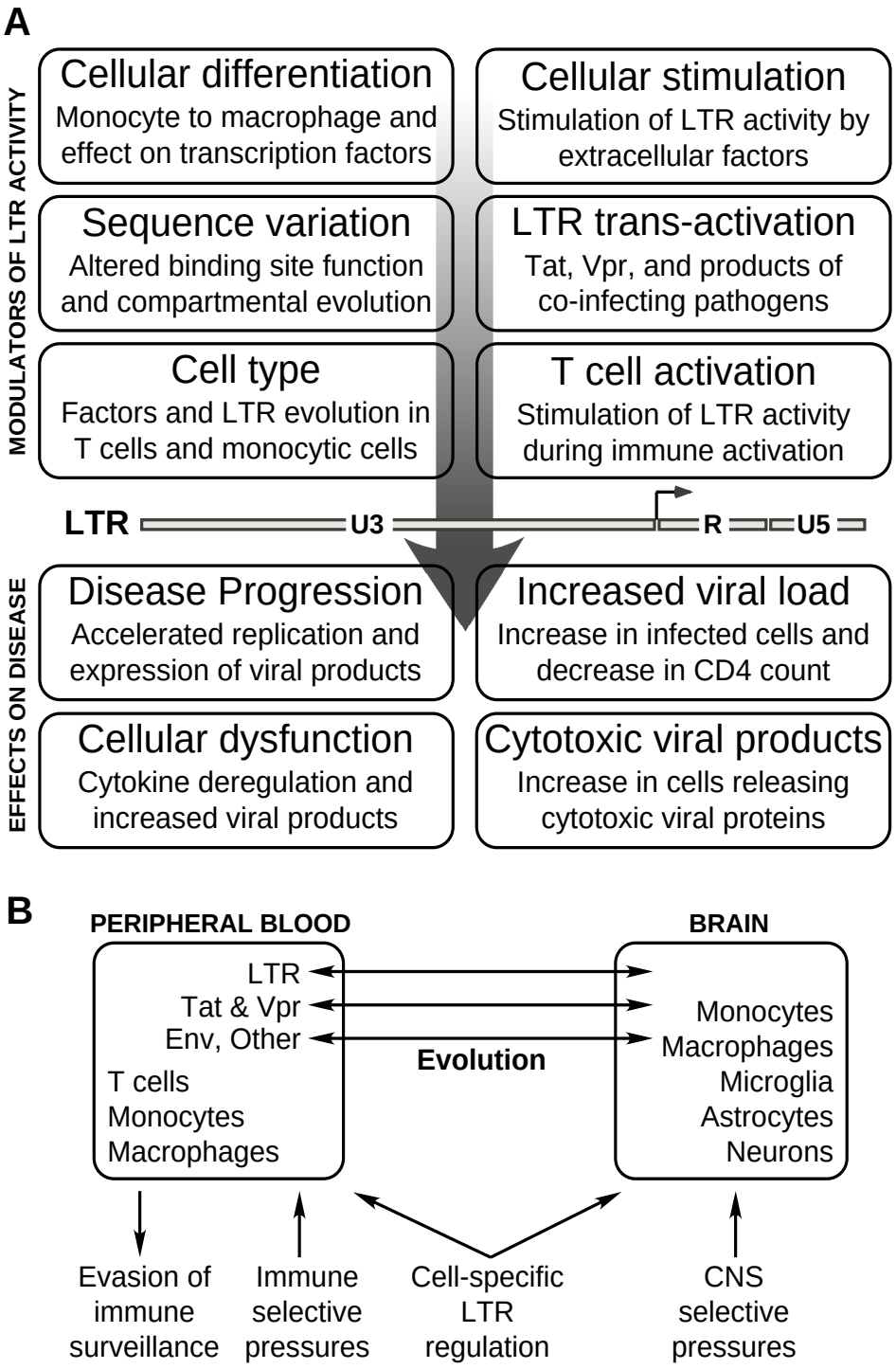


Figure 7. Involvement of the HIV-1 LTR in disease pathogenesis. *(**A**) Factors that influence HIV-1 LTR function and, subsequently, aspects of HIV-1-associated pathogenesis are summarized. (**B**) Depiction of the evolution of the LTR,* trans*-activator proteins Tat and Vpr, and other viral proteins in the context of cells of the peripheral immune system and CNS and factors that influence the course of evolution.*

blood) that selects for monocyte-tropic viruses (as determined by LTR function) in infected monocytes/macrophages that subsequently cross the BBB late in disease (117) and reside in the CNS of HIVD patients as perivascular macrophages (67).

CNS disease may also be affected by the ability of viral proteins Tat and Vpr to activate the LTR (Fig. 7B). Sequence analyses have shown that Tat genes isolated from the brains of patients with HIVD have a greater level of sequence variation than Tat genes isolated from non-demented patients (265). These nucleotide changes result in non-conserved missense and nonsense mutations that likely result in the production of Tat molecules that are defective with respect to LTR *trans*-activation, neurotoxicity, or both. If HIVD is associated with the appearance of Tat molecules defective in their ability to *trans*-activate the LTR, viral replication necessary for the genesis and progression of CNS disease may be regulated through the LTR by other mechanisms, including *trans*-activation by Vpr. Our demonstration of a direct interaction between C/EBP site I and Vpr (188) combined with the preferential appearance of specific C/EBP site variants within LTRs isolated from HIV-1-infected brain tissue (73) presents the intriguing possibility that LTR sequence variation and LTR *trans*-activation by Vpr might converge in the brain to facilitate viral replication in the brain during HIV-1-associated CNS disease. Furthermore, co-evolution of the LTR, Tat, and Vpr may result in combinations of LTR sequences and *trans*-activator proteins that further augment Vpr *trans*-activation of the LTR in the context of neurotoxic Tat molecules defective in *trans*-activation ability.

While numerous investigators have amassed a considerable body of knowledge concerning the function of the HIV-1 LTR and other related LTRs within the lentivirus genus, there are still many important questions that remain regarding the role that the LTR has in the progression of disease. For example, does reduced cell- and sequence-specific LTR activity play a role in viral evasion of immune clearance? What selective pressures within the immune system and the CNS drive LTR evolution? Are those pressures cell type- or environment-specific and are they imposed differentially on different transcription sites, as suggested by studies of binding site conservation in the blood, lymphoid tissues, and the brain (73)? Are the LTRs isolated from post-mortem, HIV-1-infected brain tissue representative of viral populations that entered the brain during the initial infection or late in disease? What are the functional and pathogenic consequences of LTR sequence compartmentalization in different tissues throughout the body (103,178) and regional differences in LTR sequence within the brain (73) and, perhaps, in other tissues? How do clade-specific differences in LTR sequence and activity (172,173,266) relate to the development of disease in geographically separated HIV-1-infected populations where different clades are prevalent? Given the complex functional interplay between factors that bind to the LTR (e.g. Sp and C/EBP factors) and proteins that activate the LTR (Tat and Vpr), do these viral *trans*-activator proteins co-evolve with the LTR in the peripheral blood, lymphoid tissues, and CNS, resulting in complementary relationships between LTR function and *trans*-activation potential that impact disease progression? What is the role of Vpr as an alternative *trans*-activator protein in disease progression (particularly in aspects of disease that involve LTR activation by the interaction of Vpr with monocyte/macrophage-specific C/EBP factors)? These are but a sampling of the important questions yet to be answered regarding the impact of LTR function on disease progression.

One means to arrive at answers to some of these important questions might be the use of animal models of lentiviral infection. Similarities between HIV-1 and SIV (e.g. the presence of C/EBP sites in the HIV-1 LTR and putative sites in the SIV LTR) could be exploited to study the contribution of LTR function to the development of immunopathogenesis and neuropathogenesis in an SIV-infected macaque model system. Structure/function LTR studies within the context of an SIV infectious molecular clone could be used to study (i) the roles of individual transcription factor binding sites (e.g. C/EBP binding sites) in the development of immunopathogenesis and neuropathogenesis, (ii) the impact of LTR sequence variation on viral clearance and immunopathogenesis, (iii) the importance of LTR sequence evolution within the brain and peripheral blood in the development of virus-associated CNS disease, and (iv) co-evolution of the LTR, Tat, and Vpr during the progression of disease.

At this writing, the AIDS epidemic is concluding its second decade. After almost 20 years of study, the scientific community has amassed a vast body of literature concerning the molecular and cellular biology, epidemiology, pathogenesis, clinical features, therapeutics, and sociology of HIV-1 infection

and AIDS. However, despite these extensive efforts, there are, as yet, no viable vaccine strategies in place for the effective prevention of infection. Furthermore, there are few chemotherapeutic options available for the treatment of individuals already infected with HIV-1, although the number of therapeutic approaches continue to grow each year. Perhaps the limited range of medical approaches to HIV-1 infection can be augmented by strategies that leverage our understanding of factors that impinge on LTR function. In fact, varying degrees of progress have been made toward the development of potential therapies that target important factors with great influence on LTR function, including Tat, Vpr, NF-κB, and others (204,267-272). However, since regulation of HIV-1 transcription by Tat and Vpr involves numerous cellular mechanisms that are also important to the normal functions of the host cell, the HIV-1 specificity of the therapy will be an important consideration. Such strategies could take advantage of the critical reliance of viral replication and the progression of disease on the expression of HIV-1 as regulated by the LTR.

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