Journal of NeuroVirology (1998) 4, 312–323
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# Sp1 and related factors fail to interact with the NF-κB-proximal G/C box in the LTR of a replication competent, brain-derived strain of HIV-1 (YU-2)

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**The HIV-1 LTR promoter proximal G/C box array has been demonstrated to function by interacting with the Sp1 transcription factor family whose members can act as either activators or repressors of transcription. In this regard, we have examined the interaction of the HIV-1 Sp binding sites with nuclear factors that are present in cell types that support HIV-1 replication, including those of lymphocytic, monocytic, and astrocytic origin. As determined by electrophoretic mobility shift (EMS) competition analyses using oligonucleotides containing the sequences of each of the Sp1 sites of HIV-1 strain LAI, the NF-κB-proximal Sp site (site III) displayed the highest binding activity compared to sites I and II with regard to Sp1 and related factors present in lymphocytic (Jurkat) and astrocytic (U-373 MG) nuclear extracts. Sp1 and two Sp3 isoforms were detected as the primary cellular constituents of DNA-protein complexes formed with the NF-κB-proximal site. Only modest differences in Sp1:Sp3 binding ratios were observed when this site was reacted with either astrocytic or lymphocytic nuclear extract. However, when nuclear extracts derived from two monocytic cell types that differ in the degree of differentiation were reacted with the HIV-1 LAI Sp site III, a large difference in Sp1 and Sp3 binding was observed. To determine if naturally occurring and replication-competent strains of HIV-1 contain base pair alterations within the Sp elements that affect the ability of the site to interact with Sp1 and related factors, a series of Sp site III variants were constructed and examined by EMS analyses. One of these sites, obtained from the published sequence of the YU-2 strain (a brain-derived macrophage tropic strain of HIV-1), displayed almost no Sp1 or Sp3 binding activity as a result of a single base pair alteration in Sp site III. This base-pair alteration, when placed in the context of an HIV-1 LAI LTR-luciferase construct, resulted in a 40–50% reduction in LTR activity in transiently transfected Jurkat and U-373 MG cells. Overall, these results suggest that specific G/C box sequence alterations present in the brain-derived HIV-1 variant YU-2, or possibly other brain-derived variants, may exhibit altered replication properties as a result of the low affinity of the NF-κB-proximal G/C box for members of the Sp transcription factor family.**

**Keywords:** HIV-1; Sp1; Sp3; LTR; CNS; astrocyte; lymphocyte; monocyte

## Introduction

Human immunodeficiency virus type 1 (HIV-1) infection of the central nervous system (CNS) can result in the clinical appearance of cognitive, behavioral, and motor abnormalities collectively termed the AIDS-associated dementia complex (ADC; Price, 1994). HIV-1-associated degradation of CNS function during ADC involves infection of neuroglial cells residing within the CNS. Although early investigations into the pathogenesis of ADC indicated that cells of monocyte-macrophage lineage (including brain microglial cells) were the predominant cell type infected within the CNS (Koenig *et al*, 1986; Wiley *et al*, 1986), other cell populations, including astrocytes, have been shown to be susceptible to viral infection, as demonstrated in recent *in vivo* investigations of HIV-1 infected patients using more sensitive detection techniques

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Received 6 October 1997; revised 5 January 1998; accepted 7 January 1998

(Nuovo *et al*, 1994; Tornatore *et al*, 1994; Bagasra *et al*, 1996). *In vitro* studies have also shown that astrocytic cells can support a more restricted level of HIV-1 infection (relative to highly productive lymphocytic infections) after a short burst of productive viral replication (Dewhurst *et al*, 1987; Tornatore *et al*, 1991; Brack-Werner *et al*, 1992).

HIV-1 replication in lymphocytes, monocytes, astrocytes, and other cell types is highly dependent, in part, on interactions between cellular transcription components and the HIV-1 long terminal repeat (LTR), which, in its 5′ position in the proviral genome, serves as the promoter for viral gene expression (reviewed in Cullen, 1991; Jones and Peterlin, 1994; Kingman and Kingman, 1996). The LTR contains numerous *cis*-acting transcriptional regulatory elements that participate in regulation of viral gene expression and replication of the viral genome under a wide variety of conditions, including cellular stimulation, division, and differentiation. These elements, which can confer both basal and inducible functions on the HIV-1 LTR, function by interacting with a variety of cellular transcription factors. Important to the regulatory function of the LTR are DNA elements located between nucleotides −77 and −45 (relative to the site of transcription initiation). This region has been shown to possess three tandem DNA elements which bind the Sp1 transcription factor (Jones *et al*, 1986). In studies using both transient expression analyses and viral replication assays, these sites have been shown to have significant roles in basal LTR function, NF-κB-mediated LTR activation, and Tat transactivation (Zeichner *et al*, 1991; Ross *et al*, 1991; Kim *et al*, 1993; Perkins *et al*, 1993; Majello *et al*, 1994).

Sp1 is a ubiquitously expressed nuclear protein that has been shown to activate transcription of a variety of promoters through its interaction with GC-rich *cis*-acting binding sites (G/C boxes). Sp1 has a strong constitutive activity, although it can be superactivated by the retinoblastoma gene product Rb (Kim *et al*, 1992). Sp1 has recently been demonstrated to be a member of a family of transcription factors that can activate or repress cellular and viral promoters. One member of the Sp family, Sp3, is also ubiquitously expressed; its isoforms can activate transcription or repress Sp-mediated transcriptional activation, depending on the context of the Sp binding sites (Birnbaum *et al*, 1995) and on the isoform of Sp3 expressed (Kennett *et al*, 1997). In contrast to the ubiquitous expression of Sp1 and Sp3, Sp4 appears to be expressed in a more limited fashion within brain tissues (Kingsley and Winoto, 1992). Sp3 and Sp4 share extensive structural similarity with regions of Sp1, including the glutamine-rich and serine/threonine-rich domains. Amino acid sequence homology is particularly strong in the DNA-binding zinc finger regions of Sp1, Sp3 and Sp4 proteins. As a result, Sp1, Sp3 and Sp4 possess similar binding affinities to cognate DNA elements (Hagen *et al*, 1992; Kingsley and Winoto, 1992). Sp2 shows greater sequence divergence within the zinc-finger domain and its DNA-binding sequence specificity has been shown to differ significantly from other Sp family members (Kingsley and Winoto, 1992). With respect to the HIV-1 LTR, Sp1 and Sp4 have been shown to be activators of transcription whereas Sp3 has been shown to repress basal and Tat-mediated activation (Majello *et al*, 1994). Sp3 repression of HIV-1 LTR activity is consistent with a general model in which Sp3 repression is dependent on the number and context of the Sp binding elements located within the promoter of interest (Birnbaum *et al*, 1995).

Our understanding of the mechanism by which Sp3 can activate or repress transcription has improved with the report that Sp3 encodes multiple proteins that differ in their capacity to activate or repress transcription (Kennett *et al*, 1997). It was reported that full-length Sp3 (a 110 kD protein) is an activator, while internally initiated isoforms of 78–80 kD, which lack sequence coding for transactivation domains, repress transcription mediated by Sp1 and full-length Sp3 (Kennett *et al*, 1997). These results suggested that competition for binding site occupancy between internally initiated Sp3 isoforms, Sp1, and full-length Sp3 could account for the repression of transcription, although it was noted that a more complex mechanism may exist since Sp3-mediated activation was more sensitive to Sp3 repression than Sp1-mediated activation (Kennett *et al*, 1997). It was suggested that regulation of the levels of internally initiated Sp3 may play an important role in cell-cycle and signal-induced transcription of G/C box-containing promoters.

Modulation of HIV-1 LTR activity by members of the Sp transcription family may also be impacted by sequence variations that arise within the Sp binding sites during *in vivo* replication. The genomic sequence of HIV-1 evolves during the course of replication due to selective pressures and nucleotide misincorporation by reverse transcriptase (Goodenow *et al*, 1989; Katz and Skalka, 1990). Naturally occurring nucleotide changes that affect the Sp binding sites affect the ability of the LTR to support both transient transcription and viral replication (Koken *et al*, 1992; Michael *et al*, 1994).

Given the recent advances in our understanding of Sp-mediated transcriptional regulation and the critical role that these elements play in LTR activation, it has become important to reevaluate the functional roles of the HIV-1 Sp elements in the regulation of cell type-, cell cycle- and signal transduction-dependent regulation of HIV-1 LTR activity. Within CNS cell populations susceptible to HIV-1 infection, differences in levels of Sp1 and each of the Sp3 isoforms may impact the ability of the HIV-1 LTR to support viral expression. Furthermore, HIV-1 Sp site sequence variations in LTR quasispecies isolated from infected CNS tissues

may affect function of these sites during viral expression in these CNS cell populations. This study was conducted to compare the HIV-1 LTR G/C box array binding activities in a series of cell types involved in HIV-1 infection and pathology (astrocytes, CD4-positive T lymphocytes, and cells of the monocyte/macrophage lineage) and to examine the impact of sequence variation in this region on Sp factor binding and LTR function.

## Results

To examine specific interactions between the HIV-1 G/C box array and Sp family members, double-stranded DNA oligonucleotides were synthesized with sequence homology to each of the three Sp sites of HIV-1 strain LAI (Figure 1a). To facilitate the identification and characterization of proteins that bind to these elements, each DNA oligonucleotide was designed to simplify the DNA-protein interactions by limiting binding to a single 10 bp site flanked by five nucleotides on either side. This resulted in the preparation of three partially overlapping oligonucleotides designated LIII, LII and LI (Figure 1a), which correspond to Sp sites III, II and I, respectively, in the LAI strain of HIV-1 (Myers *et al*, 1996). The sequence of the HIV-1 LAI G/C box array was selected for analysis since the sequence is commonly encountered in other LTRs contained within replication competent HIV-1 strains. Also shown (Figure 1b) are the published sequences of HIV-1 Sp site III for two other HIV-1 variants, YU-2 and SF1701, that contain either one or two nucleotide alterations, respectively, compared to strain LAI.

### *Sp binding to each HIV-1 Sp element is not equivalent*

To characterize the factors that bind to these elements, an LIII double-stranded oligonucleotide probe was utilized in EMS analyses with nuclear extracts prepared from astrocytic (U-373 MG) and lymphocytic (Jurkat) cell lines (Figure 1c and d). As shown, four DNA-protein complexes (designated A, B, C and D) formed when LIII probe was reacted with either U-373 MG (Figure 1c, lanes 1, 7 and 13) or Jurkat (Figure 1d, lanes 1, 7 and 13) nuclear extracts. Not shown is a significant amount of unbound probe that migrated to the bottom of each lane in the gels. To characterize the relative affinity of the factors that bind to LIII in comparison to LI and LII, unlabeled competitor oligonucleotides (LI, LII and LIII) were included in EMS reactions at a 2–100-fold molar excess over the probe concentration. In these studies, the unlabeled LIII competitor (Figure 1c, lanes 2–6) showed substantially greater ability to compete for the complexes formed with the LIII probe than did LI (Figure 1c, lanes 8–12) and LII (Figure 1c, lanes 14–18) competitors. This observation was also seen using Jurkat (Figure 1d) nuclear extracts. Also noted was the fact that complex D was competed less efficiently than complexes A, B and C, indicating that complex D involves a molecular interaction of lesser affinity or sequence specificity than the interaction observed in the formation of

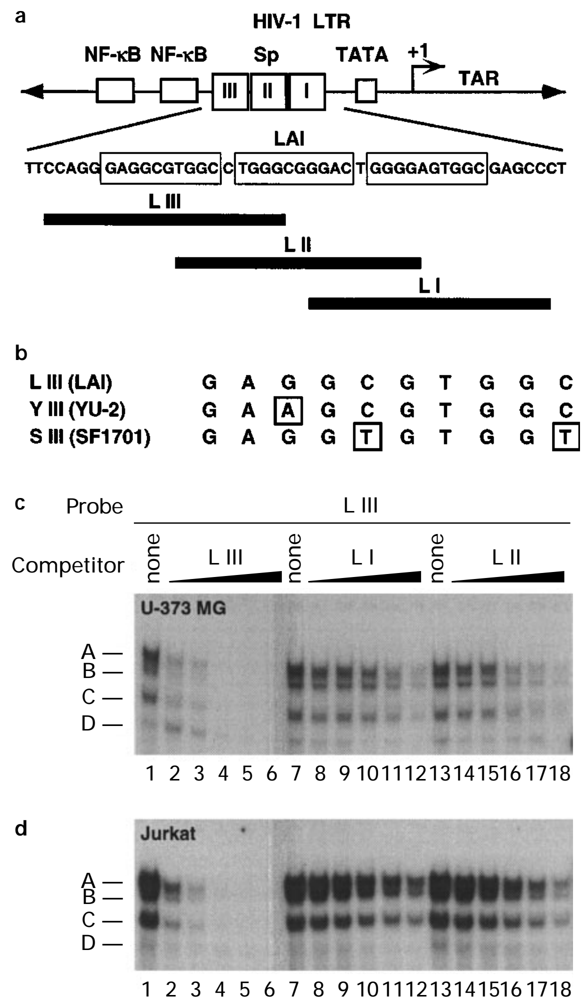


**Figure 1** (**a**) Oligonucleotides were derived from the sequence of the HIV-1 LTR Sp family binding sites. Three tandem Sp binding sites are located between the TATA box and two NF-κB binding sites within the HIV-1 LTR. Double-stranded oligonucleotides were constructed to span each of the Sp binding sites found in the LTR of strain LAI. These oligonucleotides have been designated LAI site I (LI), site II (LII) and site III (LIII). (**b**) Two additional oligonucleotides were synthesized using sequences derived from Sp binding sites III in the SF1701 (SIII) and YU-2 (YIII) LTRs (Myers *et al*, 1996). Nucleotides in SIII and YIII which differ from the sequence of site III in LAI are boxed. Nuclear proteins extracted from U-373 MG (**c**) and Jurkat (**d**) cells bind to LAI Sp binding site III with higher affinity than sites I and II. EMS analyses were conducted as described in Materials and methods. Binding reactions included 6 μg of nuclear extract, 75 000 c.p.m. (approximately 1 ng) of each radiolabeled probe, and competitor oligonucleotide (2×, 5×, 25×, 50× and 100×) where indicated. The positions of complexes A through D are indicated.

complexes A, B and C. To quantitate these results, the abundance of the complexes involving the high affinity interactions (complexes A, B and C) were summed within each lane and plotted against the competitor oligonucleotide concentration (data not shown). These results showed that the amount of competitor required for 50% complex reduction was approximatley 20- and 40-fold higher for LII and LI, respectively, compared to LIII. These results suggest that the HIV-1 LAI Sp1 site III has a significantly higher affinity than either site II or I for nuclear factors in both cell types tested. Experiments shown below clearly demonstrate that complexes A, B and C involve factors of the Sp1 family (Figure 2). These results are consistent with the studies of Jones *et al* (1986) in which it was reported that purified Sp1 binds site III with a higher affinity than sites I and II.

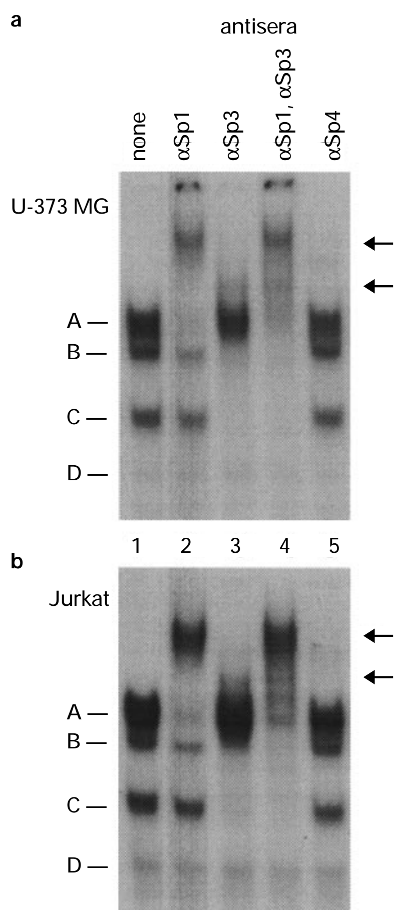


**Figure 2** Astrocytic and lymphocytic Sp family member transcription factors bind to LAI site III. EMS supershift analyses using the LAI site III probe (LIII) were performed as described in Materials and methods. Binding reactions included 6 $\mu$g of U-373 MG nuclear extract (**a**) or 6 $\mu$g of Jurkat nuclear extract (**b**) with 75 000 c.p.m. (approximately 1 ng) of radiolabeled probe, and 1 $\mu$L antisera as indicated. The arrows indicate the positions of supershifted bands.

*Sp1 and at least two Sp3 isoforms in astrocytic and lymphocytic nuclear extracts interact with HIV-1 Sp binding site III*

The U-373 MG and Jurkat nuclear factors involved in formation of complexes A, B and C were shown to be immunologically related to Sp1 and Sp3 by antibody supershift EMS analyses (Figure 2a and b). Specifically, DNA-protein complex A was shown to be supershifted by Sp1 antisera in EMS reactions utilizing either U-373 MG (Figure 2a, compare lane 2 to lane 1) or Jurkat (Figure 2b, compare lane 2 to lane 1) nuclear extracts while complexes B and C were supershifted with Sp3 antisera in EMS reactions utilizing either nuclear extract (Figure 2a and b, compare lane 3 to lane 1). Sp4 antisera reacted to a much lesser extent with complexes formed using either nuclear extract (Figure 2a and b) although a supershifted complex can be observed with longer exposures (data not shown). Consistent with results obtained with individual antisera, the combined use of Sp3 and Sp1 antisera in EMS reactions resulted in abrogation and/or supershift of a vast majority of the DNA-protein complexes formed with either nuclear extract (Figure 2a and b, compare lane 4 to lane 1). Similar types of complexes were formed using LI and LII (data not shown). These observations, in conjunction with previous studies (Kennett *et al*, 1997), indicate that complex A contains the Sp1 activator protein, complex B contains the 110 kD full-length Sp3 activator and complex C contains the truncated 78–80 kD Sp3 repressor proteins.

*Relative levels of Sp1 and Sp3 binding to LAI Sp site III differ by a small amount between astrocytic and lymphocytic nuclear extracts*

It was observed in our initial studies that the ratio of Sp1 to Sp3 may be lower in U-373 MG than in Jurkat nuclear extracts. To more accurately assess this possibility, we utilized each of these extracts in probe titration analyses under conditions in which each of the three Sp complexes could be separately quantitated (Figure 3). Increasing amounts of LIII probe (from 12 500–200 000 c.p.m.) were reacted with a constant amount of nuclear extract (6 $\mu$g). Quantitation of the EMS analyses shown in Figure 3 is displayed graphically below the autoradiograph with quantitative results obtained with U-373 MG extracts shown on the left and quantitative results obtained with Jurkat extracts shown on the right. When the amount of probe is saturating with respect to the available Sp proteins, the relative abundance of each complex should primarily reflect the relative amounts of proteins in the extracts that are capable of binding DNA. Utilizing these experimental conditions, the average ratio of Sp1 to either the large or small form of Sp3 from three independent experiments was calculated to be 20–30% higher when LIII was reacted with Jurkat nuclear extract as compared to U-373 MG extract.

Since Sp3 can function as a potent inhibitor of Sp1-activated transcription with certain *cis*-acting elements, it will be of interest to determine whether this modest difference in relative levels of astrocytic and lymphocytic Sp1 and Sp3 binding to the NF-κB-proximal Sp binding site is of functional significance.

*Naturally occurring HIV-1 Sp site III sequence variation causes alterations in DNA-protein complex formation using astrocytic and lymphocytic extracts*

Although the Sp sites of HIV-1 sequence variants remain relatively well conserved across a number of LTRs described in the Los Alamos National Laboratory Sequence Database (Myers *et al*, 1996), there are some alterations that can be found, particularly in Sp site III. Such alterations include insertion/deletion mutations of guanine nucleotides at the extreme 5′ end of site III (examples include SF33 and SF1701), cytosine (C) to thymidine (T) substitutions at position 5 of site III (examples include NL43 and SF1701), guanine (G) to adenine (A) substitutions at position 3 of site III (examples include YU-2 and YU-10c), and a variety of alterations in the 3′ region of Sp site III (examples include MAL, MANC and Z6). Based on these observations, we have proceeded to examine the impact of sequence variation in the high affinity Sp binding site III on overall binding of Sp1 and related family members as determined by EMS analyses. To initiate these studies, we compared the sequence of Sp site III in a number of HIV-1 quasispecies to identify nucleotide variations that may affect Sp binding. Four replication competent strains of HIV-1 were selected that contained one or two base pair changes within the Sp site III relative to the sequence of the LAI strain. Site III oligonucleotide probes for three of the four variant sites selected (MAL, Z6, SF1701) exhibited binding affinity for Sp1 and related family members similar to that of the LAI site III (data not shown). This is not unexpected since alterations in the 3′ half of Sp sites, as is the case with these strains of HIV-1, have less impact on Sp1 binding to the site than nucleotide changes in the central and 5′ region of the DNA element (Kriwacki *et al*, 1992). In striking contrast, one of the four site III probes exhibited a significant impairment in its ability to interact with Sp1 or related factors. This probe, designated YIII, contains the Sp site III of the brain-derived, macrophage tropic YU-2 strain of HIV-1.

EMS analyses involving U-373 MG nuclear extracts and YU-2 (YIII) and SF1701 (SIII) probes are shown in Figure 4. A parallel set of EMS reactions were also performed using Jurkat nuclear extracts (Figure 5). Quantitation of complex abundance is shown below the autoradiograph. The SIII probe demonstrated strong binding to Sp1 and Sp3 isoforms (Figures 4a and 5a, lanes 1–5) at levels similar to those seen using LIII (Figure 1c and d, lane 1; Figure 2a and b, lane 1). This evidence indicates that alterations within the core 10 bp and flanking sequence of site III of SF1701 do not strongly inhibit binding of Sp1-related factors. However, the reactions containing the YIII probe and U-373 MG nuclear extracts (Figure 4a, lanes 6–10) and Jurkat nuclear extracts (Figure 5a, lanes 6–10) were nearly devoid of Sp1 and Sp3 binding at all probe concentrations examined. Although this result is somewhat unexpected since the YIII probe contains just one base-pair change at position 3 of the Sp1 site compared to the LIII probe (Figure 1b), position 3 is normally a highly conserved guanine nucleotide and therefore it is likely that this is a critical nucleotide required for high affinity binding of Sp1 to its DNA binding site (Kriwacki *et al*, 1992). Given that site III is the strongest Sp1 site in LAI, such a large loss in binding of Sp family members due to a single nucleotide change in Sp site III of the YU-2 LTR may have a significant effect on YU-2 utilization of Sp factors during regulation of viral

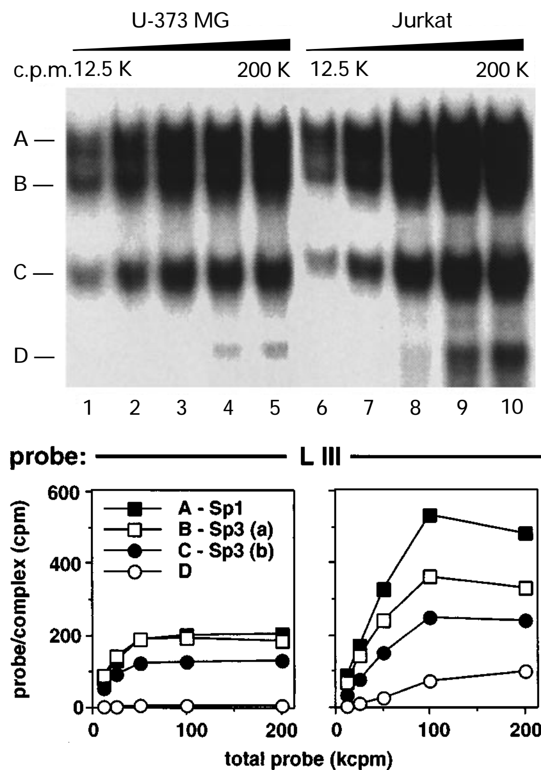


**Figure 3** Relative levels of astrocytic and lymphocytic Sp1 and Sp3 site III binding difer by small amount. EMS analyses using the LAI site III probe (LIII) were performed as described in Materials and methods. Binding reactions included 6 μg of U-373 MG or Jurkat nuclear extract and 12 500–200 000 c.p.m. (0.09–1.5 ng) of radiolabeled probe. DNA-protein complexes A through D were quantitated and graphed as c.p.m. per complex in each lane.

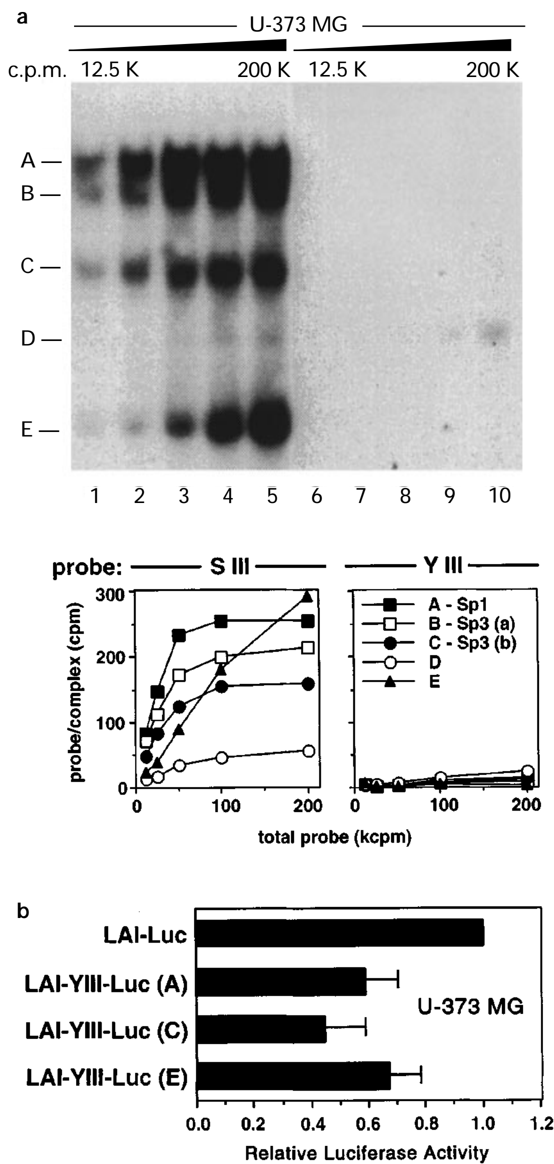


**Figure 4** Naturally-occurring Sp site III sequence variation alters DNA-protein complex formation using U-373 MG nuclear proteins. (**a**) EMS analyses using the SF1701 (SIII) and YU-2 (YIII) site III probes were performed as described in Materials and methods. Binding reactions included 6 $\mu$g of U-373 MG nuclear extract and 12 500 – 200 000 c.p.m. (0.09 – 1.5 ng) of radiolabeled probe. DNA-protein complexes A through E were quantitated and graphed as c.p.m. per complex in each lane. (**b**) Mutagenesis of the LAI LTR Sp site III to the YU-2 sequence configuration reduced LAI LTR activity by 42% in U-373 MG cells. U-373 MG cells were transfected with either LAI-Luc or LAI-YIII-Luc in conjunction with pRL-SV40 internal control vector (Promega). Three different clones of LAI-YIII-Luc (A, C and E) were utilized to ensure that any alteration in LAI LTR activity was due to the Sp site III mutation and not due to alterations that may have been generated in areas of the plasmid not sequenced. Firefly luminescence was normalized to the *Renilla* luminescence in each experiment to control for variations in transfection efficiency. Data is presented with the wild-type LTR values (LAI-Luc) arbitrarily set to 1.0 for each experiment and the relative activity of the mutagenized LTRs (LAI-YIII-Luc) normalized to this value. Error bars indicate the standard error of data obtained from four independent experiments.

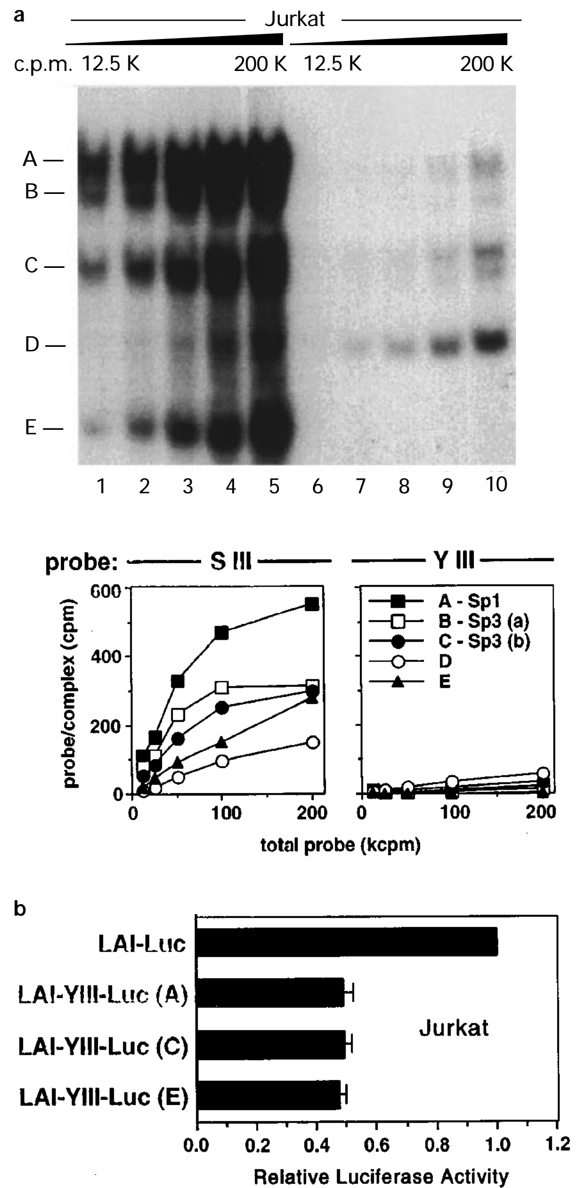


**Figure 5** Naturally-occurring Sp site III sequence variation alters DNA-protein complex formation using Jurkat nuclear proteins. (**a**) EMS analyses using the SF1701 (SIII) and YU-2 (YIII) site III probes were performed as described in Materials and methods. Binding reactions included 6 $\mu$g of Jurkat nuclear extract and 12 500 – 200 000 c.p.m. (0.09 – 1.5 ng) of radiolabeled probe. DNA-protein complexes A through E were quantitated and graphed as c.p.m. per complex in each lane. (**b**) Mutagenesis of the LAI LTR Sp site III to the YU-2 sequence configuration reduced LAI LTR activity by 52% in Jurkat cells. Jurkat cells were transfected with either LAI-Luc or LAI-YIII-Luc in conjunction with pRL-TK internal control vector (Promega). Three different clones of LAI-YIII-Luc (A, C and E) were utilized to ensure that any alteration in LAI LTR activity was due to the Sp site III mutation and not due to alterations that may have been generated in areas of the plasmid not sequenced. Firefly luminescence was normalized to the *Renilla* luminescence in each experiment to control for varying transfection efficiencies. Data is presented with the wild-type LTR values (LAI-Luc) arbitrarily set to 1.0 for each experiment and the relative activity of the mutagenized LTRs (LAI-YIII-Luc) normalized to this value. Error bars indicate the standard error of data obtained from two independent experiments.

expression. To address whether the nucleotide change in YU-2 Sp site III can affect LTR activity, an LAI LTR-luciferase construct (LAI-Luc) was compared in transient transfection analyses with a construct in which the Sp site III of the LAI LTR was changed to the YU-2 configuration (LAI-YIII-Luc; Figures 4b and 5b). To ensure that any changes in promoter activity were due to the single nucleotide alteration in Sp site III, data for three independent plasmid clones generated by the mutagenesis procedure are shown. Each of the three LTR clones was sequenced on both strands to confirm the presence of the correct mutation. These studies demonstrated that this single base-pair alteration reduced basal activity of the LAI LTR by approximately 42% in U-373 MG cells (Figure 4b) and by approximately 52% in Jurkat cells (Figure 5b). Since this base-pair change is the only nucleotide difference observed within the Sp1 elements of LAI and YU-2, these studies demonstrate that the YU-2 G/C box array is impaired in its ability to stimulate basal transcription in both U-373 MG and Jurkat cells. The fact that the functional impact of this sequence alteration was similar in both U-373 MG and Jurkat cells is consistent with our observation that the relative levels of Sp1 and Sp3 binding activity are similar in both cell types. Future studies will examine the impact of the YU-2 G/C box configuration on Tat- and NF-$\kappa$B-mediated transactivation.

In EMS analyses involving SIII, we observed the formation of an additional complex as a consequence of the two nucleotide changes in SIII. EMS reactions containing the SIII probe resulted in the formation of a substantial amount of complex E that was not detected in significant quantities in EMS reactions containing the YIII probe (Figures 4a and 5a, lanes 6–10) or LIII probe (data not shown). The cellular factor involved in this complex does not appear to be cell type-specific since it was detected in EMS reactions utilizing either U-373 MG (Figure 4a, lanes 1–5) or Jurkat (Figure 5a, lanes 1–5) nuclear extracts. In addition, it would appear that this factor binds to SIII with lower affinity than Sp1 and Sp3 since it forms abundant complexes only when large amounts of DNA probe are included in the binding reactions. Although the identity of this factor is not known, it does appear to be sequence-specific since this complex is undetectable in studies utilizing the highly related DNA probes, LIII and YIII. Although the unknown factor appears to have an affinity which is lower than that of Sp1 or Sp3 with respect to binding to the SIII probe, it could play a role *in vivo* since the intranuclear concentration of transcriptional regulatory factors *in vivo* is likely to be much higher than *in vitro* studies. Further investigation of this factor may be relevant to understanding the functional regulation of the SF1701 LTR.

### *Difference in LAI site III binding identified between Sp-related factors in U-937 and THP-1 monocytic cell lines*

Since cells of the monocyte/macrophage lineage are one of the principle cell types targeted by HIV-1 during invasion of the CNS and during disease progression, we have also examined relative levels of Sp1 and related factors that are capable of interacting with the LTR G/C box array in this cell type (Figure 6). As *in vitro* models of the macrophage lineage, we have selected two cell lines which differ in their overall level of differentiation (U-937 and the more mature THP-1). As shown utilizing Jurkat and U-373 MG nuclear extracts and LIII, DNA-protein complexes formed with U-937 and THP-1 nuclear extracts also appear to be comprised primarily of Sp1 and Sp3 (Figure 6, lanes 1 and 2 compared to lanes 3 and 4). Of greatest interest is the observation that the abundance of the complexes containing Sp1 activator and full-length Sp3 activator (complexes A+B) relative to truncated Sp3 inhibitor (complex C) in the reactions containing the THP-1 nuclear extract appears noticeably greater in comparison to other EMS reactions utilizing nuclear extracts derived from Jurkat, U-373 MG, and U-937 cells. Quantitation of this

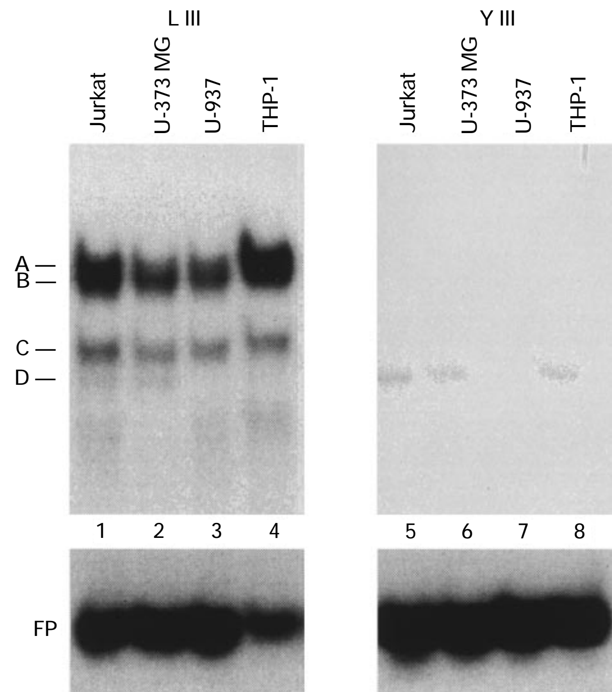


**Figure 6** Astrocytic, lymphocytic, and monocytic Sp1 and Sp3 bind LAI and YU-2 site III oligonucleotide probes at different ratios. EMS analyses using the LAI (LIII) and YU-2 (YIII) site III probes were performed as described in Materials and methods. Binding reactions included 6 $\mu$g of nuclear extract and 100 000 c.p.m. (approximately 0.7 ng) of radiolabeled probe. The positions of complexes A through D are indicated. The free probe (FP) is shown to illustrate the lack of complex formation using the YIII probe despite an abundance of free probe.

(A+B)/C ratio yielded values of 3.4 for Jurkat, 2.7 for U-373 MG, 2.7 for U-937 and 4.7 for THP-1. In addition, as shown in Figure 6 (lanes 5–8), neither of the monocytic nuclear extracts displayed strong binding to the YIII probe. Additional studies will focus on identifying any differences in Sp1/Sp3 expression in these monocytic cell lines and determining the relationship between Sp1/Sp3 expression, monocyte differentiation, and LTR regulation.

## Discussion

It has been well established that the Sp1 sites in the HIV-1 LTR play a major role in both basal and Tat-induced LTR transcriptional activity (Harrich *et al*, 1989; Ross *et al*, 1991; Moses *et al*, 1994; Sune and Garcia-Blanco, 1995). Sp1, Sp3 and Sp4 have been implicated in directing cellular control of the LTR (Majello *et al*, 1994). However, it has not been clearly demonstrated how different Sp1 family members interface to control LTR activity in different cell types that serve as hosts for HIV-1 replication. Recent studies demonstrating that Sp3 transcriptional activity can be regulated by the alternative use of internal translational initiation sites (Kennett *et al*, 1997) suggest a more complex model of transcriptional regulation by Sp family members. It has been speculated that alterations in the protein and/or DNA binding activities of internally initiated Sp3 isoforms may account for cell cycle- or signal-induced expression of G/C box regulated promoters (Kennett *et al*, 1997). In line with this hypothesis is recent information demonstrating that relative levels of Sp1 and Sp3 can be altered during cellular differentiation (Apt *et al*, 1996). Our studies were initiated to assess the interaction of Sp1 and related family members present in different cell types implicated in HIV-1 replication (including CD4-positive T lymphocytes, astrocytes, and cells of monocyte/macrophage lineage) with the HIV-1 LTR G/C box array, and to determine how Sp site III sequence variation may impact Sp1-related binding to this important HIV-1 regulatory sequence.

Our studies utilizing single HIV-1 Sp site DNA oligonucleotides demonstrated that the three Sp binding sites of HIV-1 strain LAI are not equal in their ability to interact with lymphocytic and astrocytic Sp1 family members. Specifically, LAI Sp site III exhibits greater Sp1 and Sp3 binding activity than sites I and II. This is not surprising since there is evidence that the Sp sites closest to the NF-$\kappa$B site are most important for LTR activity (Li *et al*, 1994). The functional importance of differential affinity of the three HIV-1 Sp sites of LAI, or any other HIV-1 strain, is not clear. However, it could be speculated that the region as a whole may require specific nucleotide structures to facilitate binding of proteins to all three sites concurrently. Alternatively, each site may provide unique regulatory elements that function by binding factors other than Sp1 family members in specific cell types or during activation of specific signal transduction pathways. Many other replication competent variants of HIV-1 do not share complete sequence similarity with LAI, and these differences in sequence may alter the ability of the site variants to interact with Sp family members and potentially other G/C box binding proteins. This scenario is best exemplified with site III in HIV-1 strain YU-2 where one base pair change at position three of the 10 bp element dramatically reduced Sp binding.

Of the cell types examined, the HIV-1 Sp site III complexes were comprised predominantly of Sp1 and two or more Sp3 isoforms. The presence of both a slow migrating Sp3 complex and a fast migrating Sp3 complex is consistent with studies demonstrating that internally initiated Sp3 (78–80 kD) migrates as the faster complex, whereas full-length Sp3 (110 kD) migrates more slowly in DNA-protein binding assays (Kennett *et al*, 1997). This indicates that all of these DNA elements may be targets for positive regulation via Sp1 and full-length Sp3, and negative regulation via internally translated Sp3 isoforms. In addition to complexes containing Sp1 and Sp3, we did detect other complexes in our DNA-protein interaction studies. However, the functional impact of these factors is unclear since they require a significantly higher DNA target concentration to bind the Sp sites.

Although we did detect moderate cell type differences in binding studies examining the ratios of Sp1 and Sp3 isoforms in the lymphocytic and astrocytic cell lines tested, it is unlikely that these small differences play a major role in altering LTR activity. Of greater interest are the observations using nuclear extracts derived from cell lines of monocyte lineage. Our EMS analyses (Figure 6) indicate that the differentiated phenotype of two monocytic lineage cell lines correlates with a 75% increase in the ratio of Sp activators (Sp1 and full-length Sp3) to the truncated Sp3 repressor forms in THP-1 compared to U-937 cells. Studies are in progress to determine if this difference is due to alterations in abundance or affinity of Sp1 or one of the Sp3 isoforms. This observation indicates the need to further examine Sp1 and Sp3 levels in monocytes and to determine what happens to these levels during monocyte differentiation.

Our studies utilizing Sp sites of selected replication competent HIV-1 quasispecies demonstrate that Sp binding to site III of the HIV-1 LTR can be significantly impaired in an infectious strain of HIV-1. We demonstrated that strain YU-2, a replication competent, brain-derived isolate of HIV-1 (Li *et al*, 1991), contains a single base pair change in site III (relative to LAI) that almost completely eliminates the binding of Sp1-related factors to the 5′ Sp1 site.

It is interesting that HIV-1 strain YU-2 contains a G/C box array that is not only impaired in its ability to bind Sp-related factors, but is also significantly impaired in its ability to support basal LTR activity, at least within the context of the LAI LTR. Some studies support the possibility that functional redundancy within the Sp region may allow for equal LTR activity when one of the three Sp sites is altered to inhibit Sp binding (Perkins *et al*, 1993). Our studies do not negate the model of functional redundancy since we have not looked at LTRs which have all three Sp sites mutated to block binding. However, our studies do indicate that inhibiting binding to the 5′ Sp site using the YIII sequence configuration can result in a reproducible reduction in basal LTR activity of approximately 50% in the two cell lines tested. Although our studies have not addressed the impact of transcriptional regulatory elements outside the G/C box region, they strongly suggest that the G/C box array of YU-2 plays a different role in regulating LTR activity in this variant than the G/C box array in LAI. The presence of the YU-2 Sp site III sequence configuration is not restricted to strain YU-2; it has also been found in the LTRs of strain YU-10c, as well as in PCR amplified LTRs in nervous system and lung tissue of an individual that had died of AIDS (Ait-Khaled *et al*, 1995). Differences in the functional role of these critical elements due to sequence variation may reflect differences in the cell types infected by specific HIV-1 strains since different cell types and the degree of cellular differentiation may yield different levels of Sp1-related factors. Further investigations into Sp1- and Sp3-mediated LTR regulation in monocytes may enhance our understanding of LTR regulation during infection of quiescent monocytes and activated macrophages, and our general knowledge of the roles that G/C box elements play in the process of cellular differentiation.

The YU-2 Sp site III sequence variant may also result in the generation of a new transcription factor binding site, or reduced competition between Sp element-binding factors by severely reducing the affinity of Sp1 factors for site III. The thyroid hormone receptor, for instance, has been shown to interact with the HIV-1 Sp elements in a manner mutually exclusive with Sp1 (Desai-Yajnik and Samuels, 1993). It is possible that a point mutation could have little or no effect on thyroid hormone receptor binding while abrogating Sp1 interactions, thus increasing the potential for thyroid hormone receptor function through this region. In addition, sequence analysis of YU-2 Sp site III using the TRANSFAC transcription factor database (Wengender *et al*, 1997) shows that the YU-2 base-pair change may create a binding site for the Ets family of transcription factors through the GGGAAG motif of YU-2 Sp site III. This 6 bp motif matches a described Ets-2 binding site located within the cdc-2 promoter (Wen *et al*, 1995). Our current DNA-protein interaction studies are not consistent with this hypothesis since we have demonstrated that the formation of DNA-protein complexes involving the YU-2 site III oligonucleotide was minimal compared to the LAI Sp1 binding site III. However, it is possible that transcriptional regulatory proteins such as Ets-2 may function through YU-2 Sp site III in other cell types, during different stages of cellular differentiation, or during the activation of specific signal transduction pathways.

We also demonstrated the formation of an additional DNA-protein complex as a consequence of base pair changes within Sp site III. The Sp site III of the SF1701 strain of HIV-1, in addition to binding Sp1 and Sp3, shows an ability to form a unique DNA-protein complex with a nuclear factor of both astrocytic and lymphocytic origin not seen with Sp site III of either LAI or YU-2. The formation of the SF1701-specific complex occurs most efficiently under conditions in which Sp1 and Sp3 factor binding is saturated, indicating that Sp1 and Sp3 are likely to prevent this factor from binding to the SF1701 Sp site III under conditions where target DNA is limiting. Since it is not yet clear how these DNA-protein interactions occur *in vivo*, we cannot yet make a strong prediction about the binding and function of this protein *in vivo*. Further work will be required to identify this factor and examine its role in the regulation of the SF1701 LTR.

## Materials and methods

*Cell culture and nuclear extract preparation*

The human U-373 MG (astrocytic; ATCC HTB-17) cell line was cultured in Dulbecco's Minimal Essential Medium. The human Jurkat (lymphocytic; ATCC TIB-152), U-937 (monocytic; ATCC CRL-1593), and THP-1 (monocytic; ATCC TIB-202) cell lines were grown in RPMI 1640 media. All media were supplemented with 10% heat-inactivated fetal bovine serum, antibiotics (penicillin, streptomycin, and kanamycin at 0.04 mg/ml each), 0.3 mg/ml L-glutamine, and 0.05% sodium bicarbonate. All cell lines were maintained at 37°C and 90% relative humidity under 5% $CO_2$. Nuclear extracts were prepared (Dignam *et al*, 1983) and quantitated for protein concentration (Bradford, 1976) as described previously.

*Oligonucleotide probe synthesis and labeling*

Complementary single-stranded oligonucleotides were synthesized (Marcomolecular Core Facility, Penn State College of Medicine, Hershey, PA) and annealed by heating to 100°C followed by gradual cooling to room temperature. The resulting double-stranded oligonucleotides were gel purified and radiolabeled using T4 polynucleotide kinase (Gibco BRL, Gaithersburg, MD) and [$\gamma$-$^{32}$P]ATP.

*EMS analyses*
EMS analyses were based on previously described techniques (Garner and Revzin, 1981). Radiolabeled probe was incubated with nuclear extract and 1 μg poly (dI-dC) for 20 min at 30°C. DNA-protein complexes were resolved by electrophoresis for 3–5 h at 125 V and 4°C in a 4% nondenaturing polyacrylamide gel, which was subsequently dried at 80°C for 45 min and subjected to autoradiography. DNA-protein complex formation was quantitated using a Betascope imaging system (Marcomolecular Core Facility, Penn State College of Medicine, Hershey, PA). Competition EMS analyses were conducted by adding a molar excess of unlabeled homologous or heterologous oligonucleotide probe. Antibody supershift EMS analyses were performed by adding the antisera to the binding reactions 10 min prior to loading the gel. Antisera specific to Sp1, Sp3 and Sp4 were obtained commercially (Santa Cruz Biotechnology, CA).

*Plasmids and site-directed mutagenesis*
The wild-type LAI LTR, obtained from Dr Maureen Goodenow (University of Florida), was cloned into the pGL3-Basic Vector (Promega) to construct LAI-Luc. LAI-Luc was used as a template for site-directed mutagenesis using the QuickChange mutagenesis kit (Stratagene) to construct LAI-YIII-Luc. LAI-YIII-Luc has a single G to A nucleotide substitution at position 3 in the NF-κB-proximal Sp site giving this site the configuration published for the YU-2 LTR (GA$\underline{\text{A}}$GCGTGGC). Sequencing was conducted on a number of clones derived from the mutagenesis procedure; three clones (A, C and E) which had the correct nucleotide substitution within the LAI LTR were selected for use in transfection studies.

*Transient transfections and luciferase assays*
Transient transfections were performed using a liposome-mediated delivery system. For Jurkat cells, exponentially growing cultures were aliquoted at $10^6$ cells per 0.8 ml Optimem immediately prior to transfection. Jurkat cells were transfected with 0.3 μg of either LAI-Luc or LAI-YIII-Luc in conjunction with 0.025 μg of pRL-TK *Renilla* internal control vector (Promega) for 3–5 h at 37°C in the presence of 10 μg Lipofect-AMINE (Gibco BRL) and 1 ml of Optimem. For U-373 MG cells, exponentially growing cells ($3 \times 10^5$) were transfected in 1 ml Optimem including 1 μg of either LAI-Luc or LAI-YIII-Luc in conjunction with 0.025 μg of the pRL-SV40 *Renilla* internal control vector (Promega) for 3 h. Cultures were permitted to grow for a total of 24 h after the start of the transfection. Cell extracts were harvested following the instructions of the Dual Luciferase Assay Kit (Promega). Firefly luminescence was normalized to the *Renilla* luminescence to control for variability in transfection efficiency. Data is presented with the wild-type LTR values (LAI-Luc) arbitrarily set to 1.0 for each experiment and the relative activity of the mutagenized LTRs (LAI-YIII-Luc) normalized to this value. Error bars indicate the standard error of data obtained from two (Jurkat) or four (U-373 MG) independent experiments.

## Acknowledgements

We acknowledge the Penn State College of Medicine Macromolecular Core Facility for the synthesis of oligonucleotides. These studies were supported by grants from the National Institute of Neurological Disorders and Stroke, National Institutes of Health (NS 32092 and NS 27405). We would also like to acknowledge the Julius H. Caplan Foundation for financial support in honor of Helen Caplan.

## References

Ait-Khaled M, McLaughlin JE, Johnson MA, Emery VC (1995). Distinct HIV-1 long terminal repeat quasispecies present in nervous tissues compared to that in lung, blood and lymphoid tissues of an AIDS patient. *AIDS* **9:** 675–683.

Apt D, Watts RM, Suske G, Bernard HU (1996). High Sp1/Sp3 ratios in epithelial cells during epithelial differentiation and cellular transformation correlate with the activation of the HPV-16 promoter. *Virology* **224:** 281–291.

Bagasra O, Lavi E, Bobroski L, Khalili K, Pestaner JP, Tawadros R, Pomerantz RJ (1996). Cellular reservoirs of HIV-1 in the central nervous system of infected individuals: identification by the combination of in situ polymerase chain reaction and immunohistochemistry. *AIDS* **10:** 573–585.

Brack-Werner R, Kleinschmidt A, Ludvigsen A, Mellert W, Neumann M, Herrmann R, Khim MC, Burny A, Muller-Lantzsch N, Stavrou D, Erfle V (1992). Infection of human brain cells by HIV-1: restricted virus production in chronically infected human glial cell lines. *AIDS* **6:** 273–285.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:** 248–254.

Birnbaum MJ, van Wijnen AJ, Odgren PR, Last TJ, Suske G, Stein GS, Stein JL (1995). Sp1 trans-activation of cell cycle regulated promoters is selectively repressed by Sp3. *Biochemistry* **34:** 16503–16508.

Cullen BR (1991). Regulation of human immunodeficiency virus replication. *Annu Rev Micro* **45:** 219–250.

Desai-Yajnik V, Samuels HH (1993). The NF-kappa B and Sp1 motifs of the human immunodeficiency virus type 1 long terminal repeat function as novel thyroid hormone response elements. *Mol Cell Biol* **13:** 5057–5069.

Dewhurst S, Bresser J, Stevenson M, Sakai K, Evinger-Hodges MJ, Volsky DJ (1987). Susceptibility of human glial cells to infection with human immunodeficiency virus (HIV). *FEBS Lett* **213:** 138–143.

Dignam JD, Lebovitz RM, Roeder RG (1983). Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res* **11:** 1475–1489.

Garner MM, Revzin A (1981). A gel electrophoresis method for quantifying the binding of proteins to specific DNA regions: application to components of the Escherichia coli lactose operon regulatory system. *Nuc Acids Res* **9:** 3047–3060.

Goodenow MM, Huet T, Saurin W, Kwok S, Sninsky J, Wain-Hobson S (1989). HIV-1 isolates are rapidly evolving quasispecies: evidence for viral mixtures and preferred nucleotide substitutions. *J Acquir Immune Defic Syndr Hum Retrovirol* **2:** 344–352.

Hagen G, Muller S, Beato M, Suske G (1992). Cloning by recognition site screening of two novel GT box binding proteins: a family of Sp1 related genes. *Nucleic Acids Res* **20:** 5519–5525.

Harrich D, Garcia J, Wu F, Mitsuyasu R, Gonazalez J, Gaynor R (1989). Role of Sp1-binding domains in in vivo transcriptional regulation of the human immunodeficiency virus type 1 long terminal repeat. *J Virol* **63:** 2585–2591.

Jones KA, Kadonaga JT, Luciw PA, Tjian R (1986). Activation of the AIDS retrovirus promoter by the cellular transcription factor, Sp1. *Science* **232:** 755–759.

Jones KA, Peterlin BM (1994). Control of RNA initiation and elongation at the HIV-1 promoter. *Annu Rev Biochem* **63:** 717–743.

Katz RA, Skalka AM (1990). Generation of diversity in retroviruses. *Annu Rev Genet* **24:** 409–445.

Kennett SB, Udvadia AJ, Horitz JM (1997). Sp3 encodes multiple proteins that differ in their capacity to stimulate or repress transcription. *Nucleic Acids Res* **25:** 3110–3117.

Kim SJ, Onwuta US, Lee YI, Li R, Botchman MR, Robbins PD (1992). The retinoblastoma gene product regulates Sp1-mediated transcription. *Mol Cell Biol* **12:** 2455–2463.

Kim JY, Gonzalez-Scarano F, Zeichner SL, Alwine JC (1993). Replication of type 1 human immunodeficiency viruses containing linker substitution mutations in the −201 to −130 region of the long terminal repeat. *J Virol* **67:** 1658–1662.

Kingman SM, Kingman AJ (1996). The regulation of human immunodeficiency virus type-1 gene expression. *Eur J Biochem* **240:** 491–507.

Kingsley C, Winoto A (1992). Cloning of GT box-binding proteins: a novel Sp1 multigene family regulating T-cell receptor gene expression. *Mol Cell Biol* **12:** 4251–4261.

Koenig S, Gendelman HE, Orenstein JM, Dal Canto MC, Pezeshkpour GH, Yungbluth M, Janotta F, Aksamit A, Martin MA, Fauci AS (1986). Detection of AIDS virus in macrophages in brain tissue from AIDS patients with encephalopathy. *Science* **233:** 1089–1093.

Koken SE, van Wamel JL, Goudsmit J, Berkhout B, Geelen JL (1992). Natural variants of the HIV-1 long terminal repeat: analysis of promoters with duplicated DNA regulatory motifs. *Virology* **191:** 968–972.

Kriwacki RW, Schultz SC, Steitz TA, Caradonna JP (1992). Sequence-specific recognition of DNA by zinc-finger peptides derived from the transcription factor Sp1. *Proc Natl Acad Sci USA* **89:** 9759–9763.

Li Y, Kappes JC, Conway JA, Price RW, Shaw GM, Hahn BH (1991). Molecular characterization of human immunodeficiency virus type 1 cloned directly from uncultured human brain tissue: identification of replication-competent and -defective viral genomes. *J Virol* **65:** 3973–3985.

Li Y, Mak G, Franza BR Jr (1994). *In vitro* study of functional involvement of Sp1, NF-kappa B/Rel, and AP1 in phorbol 12-myristate 13-acetate-mediated HIV-1 long terminal repeat activation. *J Biol Chem* **269:** 30616–30619.

Majello B, De Luca P, Hagen G, Suske G, Lania L (1994). Different members of the Sp1 multigene family exert opposite transcriptional regulation of the long terminal repeat of HIV-1. *Nucleic Acids Res* **22:** 4914–4921.

Michael NL, D'Arcy L, Ehrenberg PK, Redfield RR (1994). Naturally occurring genotypes of the human immunodeficiency virus type 1 long terminal repeat display a wide range of basal and tat-induced transcriptional activities. *J Virol* **68:** 3163–3174.

Moses AV, Ibanez C, Gaynor R, Ghazal P, Nelson JA (1994). Differential role of long terminal repeat control elements for the regulation of basal and Tat-mediated transcription of the human immunodeficiency virus in stimulated and unstimulated primary human macrophages. *J Virol* **68:** 298–307.

Myers G, Foley B, Mellors JW, Korber B, Wain-Hobson S, Jeang K-T, (eds) (1996). *Human retroviruses and AIDS 1996. A* compilation and analysis of nucleic and amino acid sequences. Los Alamos National Laboratory, Los Alamos, NM.

Nuovo GJ, Gallery F, MacConnell P, Braun A (1994). In situ detection of PCR-amplified HIV-1 nucleic acids and tumor necrosis factor alpha RNA in the central nervous system. *Am J Pathol* **144:** 659–666.

Perkins ND, Edwards NL, Duckett CS, Agranoff AB, Schmid RM, Nabel GJ (1993). A cooperative interaction between NF-kappa B and Sp1 is required for HIV-1 enhancer activation. *EMBO J* **12:** 3551–3558.

Price RW (1994). Understanding the AIDS Dementia Complex (ADC): The challenge of HIV and its effects on the central nervous system. In: *HIV, AIDS and the Brain*. Price RW, Perry SW III, (eds). Raven Press: New York, pp 1–45.

Ross EK, Buckler-White AJ, Rabson AB, Englund G, Martin MA (1991). Contribution of NF-kappa B and Sp1 binding motifs to the replicative capacity of human immunodeficiency virus type 1: distinct patterns of viral growth are determined by T-cell types. *J Virol* **65:** 4350–4358.

Sune C, Garcia-Blanco MA (1995). Sp1 transcription factor is required for in vitro basal and Tat-activated transcription from the human immunodeficiency virus type 1 long terminal repeat. *J Virol* **69:** 6572–6576.

Tornatore C, Nath A, Amemiya K, Major EO (1991). Persistent human immunodeficiency virus type 1 infection in human fetal glial cells reactivated by T-cell factor(s) or by the cytokines tumor necrosis factor alpha and interleukin-1 beta. *J Virol* **65:** 6094–6100.

Tornatore C, Chandra R, Berger JR, Major EO (1994). HIV-1 infection of subcortical astrocytes in the pediatric central nervous system. *Neurology* **44:** 481–487.

Wen SC, Ku DH, De Luca A, Claudio PP, Giordano A, Calabretta B (1995). ets-2 regulates cdc2 kinase activity in mammalian cells: coordinated expression of cdc2 and cyclin A. *Exp Cell Res* **217:** 8–14.

Wiley CA, Schrier RD, Nelson JA, Lampert PW, Oldstone MBA (1986). Cellular localization of human immunodeficiency virus infection within the brains of acquired immune deficiency syndrome patients. *Proc Natl Acad Sci USA* **83:** 7089–7093.

Wingender E, Kel AE, Kel OV, Karas H, Heinemeyer T, Dietze P, Knuppel R, Romaschenko AG, Kolchanov NA (1997). TRANSFAC, TRRD and COMPEL: towards a federated database system on transcriptional regulation. *Nucleic Acids Res* **25:** 265–268.

Zeichner SL, Kim JY, Alwine JC (1991). Linker-scanning mutational analysis of the transcriptional activity of the human immunodeficiency virus type 1 long terminal repeat. *J Virol* **65:** 2436–2444.