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Short Communication

HIV-1 infection and the developing nervous system: lineage-specific regulation of viral gene expression and replication in distinct neuronal precursors

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Neurologic abnormalities are common in HIV-1 infected patients and often represent the dominant clinical manifestation of pediatric AIDS. Although the neurological dysfunction has been directly related to CNS invasion by HIV-1, the pathogenesis of neurologic disorders remains unclear. Microglia and macrophages are major HIV-1 targets in the brain, whereas HIV-1 infected neurons or glial cells have been rarely reported. This suggests that indirect mechanisms may account for the severe neuronal damage observed in these patients. Nevertheless, immature, mitotically active neuronal and glial cells, which are present during fetal development, are susceptible to HIV-1 infection and replication *in vitro*, suggesting that HIV-1 infection during organ development may present unique features. To better characterize virus-host cells interactions in the developing CNS, we have examined the susceptibility of embryologically and biochemically distinct neuronal cell lines to HIV-1 infection. Here we show that mitotically active, immature neurons of distinct lineages, have different susceptibilities to HIV-1 infection and replication and different abilities to support viral gene expression. Mutational analysis of HIV-1 LTR reveals that a region of the viral promoter between nucleotide −255 to −166 is responsible for most quantitative and qualitative differences in viral transactivation among different neuroblasts. This suggests that specific regions of the viral promoter and cellular factors, either lineage- or differentiation-dependent, which bind to those regions, may contribute to control the levels of virus replication and possibly restrict the viral tropism in the developing brain. This may contribute to the establishment of a virus reservoir in the immature CNS and participate by either direct or indirect mechanisms to the severity of the AIDS-related pediatric neurological dysfunction.

Keywords: gene regulation; HIV-1; CNS; development cell cycle; pediatric aids

Introduction

Degenerative neurological abnormalities are common in human immunodeficiency virus type-1 (HIV-1) infected patients (reviewed by Atwood *et al*, 1993; Janssen *et al*, 1989) and represent a major manifestation of pediatric AIDS (Belman *et al*, 1985; Epstein *et al*, 1985, 1986; reviewed by Calvelli *et al*, 1990). Although the neuropathology has been directly related to HIV-1 invasion of the CNS (Ho *et al*, 1985; reviewed by Budka, 1989), the pathogenic mechanism(s) of the neurologic disorders are still unknown. Immune-competent cells (microglia, macrophages and lymphocytes) are major targets of HIV-1 infection in the brain (Elbott *et al*, 1989; Koenig *et al*, 1986; Shaw *et al*, 1985; Pumarola-Sune *et al*, 1987; Watkins *et al*, 1990; Wiley *et al*, 1986, 1990), suggesting that indirect mechanisms of neural damage may account for the neurological impairment observed in AIDS patients.

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In HIV-1 infected children, however, CNS disorders often accompany a vertically acquired infection (Calvelli *et al*, 1990; Lyman *et al*, 1990) and are characterized by a rapid onset of symptoms, rare occurrence of opportunistic infections and neoplasms, severe neurodevelopment, retardation, encephalopathy and cortical atrophy (Belman *et al*, 1985; Epstein *et al*, 1985; Reviewed by Budka, 1989). Whether virus- or host-factors may influence the severity of the disease in newborn and infants compared to that seen in adults is unclear. One important difference that may explain this apparent discrepancy is the timing of HIV-1 infection. In fact, mother to infant transmission can occur as early as the second trimester of pregnancy (Lyman *et al*, 1990). At this time the nervous system is still developing and is characterised by the presence of mitotically active, immature neuronal and glial cells which, influenced by extrinsic regulatory signals, are developing toward the mature state. Recent studies indicated that immature neuronal and glial cells can be susceptible to HIV-1 infection and permissive to virus replication (Ensoli *et al*, 1995; Nuovo *et al*, 1994; Sharpless *et al*, 1992; Tornatore *et al*, 1994; Truckenmiller *et al*, 1993). Thus, HIV-1 infection during CNS development may differ from that observed in the mature brain. To better characterise virus-host cells interactions during CNS development and maturation, we investigated whether embryologically and biochemically distinct neuroblastic cell lines, which represent some of the heterogeneity of neuronal precursors in the developing nervous system, are susceptible to HIV-1 infection, and examined viral gene expression and replication in these cells.

HIV-1 infection and replication in embryologically and biochemically distinct neuroblastic cell lines

To verify the degree of susceptibility of different neuronal precursors to infection by HIV-1 and their ability to support viral replication, infection experiments were performed with the primary sensory neuronal cell culture FNC-B4 (Vannelli *et al*, 1995) and two neuroblastoma-derived cell lines (SH-SY5Y and NGP) with different biochemical and functional phenotypes (Azar *et al*, 1994; Ciccarone *et al*, 1989; Cooper *et al*, 1991; Schwab *et al*, 1983; Tsokos *et al*, 1987). The primary human olfactory neuronal long-term cell culture FNC-B4 has been isolated, cloned and propagated *in vitro* from the olfactory neuroepithelium of an 8-week-old normal human fetus (Ensoli *et al*, 1995; Vannelli *et al*, 1995). These cells, which express both neuronal proteins and olfactory genes, are representative of normal sensory neuronal precursors (Ensoli *et al*, 1994b; Kubota *et al*, 1994; Vannelli *et al*, 1995). Cryogenically preserved, early passages of FNC-B4 cells were used in the present study. The neuroblastoma-derived NGP_2 and SH-SY5Y are clonal derivatives from primary tumor cell lines and are composed of homogeneous neuroblastic populations which have been previously established, cloned and propagated *in vitro* (Azar *et al*, 1994; Ciccarone *et al*, 1989; Schwab *et al*, 1983; Zehnbauer *et al*, 1988). The biochemical, phenotypical and morphological data suggest a close resemblance with their normal sympathetic and medullary counterparts, respectively, of the developing nervous system (Azar *et al*, 1994; Ciccarone *et al*, 1989; Cooper *et al*, 1991; Tsokos *et al*, 1987). In addition, SH-SY5Y cells can be induced to morphologically (neurite extension) and biochemically (differential expression of neurofilament proteins and neurotransmitters) differentiate *in vitro* under appropriate stimuli (i.e. phorbol esters, retinoic acid) (Pahlman *et al*, 1983; Thiele *et al*, 1985; Matsumoto *et al*, 1995). This process involves virtually all the cells present in these cultures indicating they are composed of a relatively homogeneous population of neuroblasts consistent with sympathetic precursors. Thus, the three cell lines mimic some of the biochemical and functional heterogeneity characteristic of the developing nervous system.

The cells were cultured in Coon's modified F12 medium supplemented with 10% fetal bovine serum in 5% CO_2 atmosphere at 37°C (Vannelli *et al*, 1995). HIV-1 infection was performed by a cell-free technique as previously described (Ensoli *et al*, 1994a). Briefly, cells were seeded in six-well tissue culture plates in Coon's modified F12 medium supplemented with 10% Fetal Bovine Serum and antibiotics. Twenty-four hours later, the medium was removed and the cells were incubated with 0.5 ml of fresh medium containing 2.5×10^5c.p.m./ml of Reverse Transcriptase Activity of the macrophagotropic HIV-1/Ba-L strain (Advanced Biotechnologies Inc, Columbia, MD). After 2 h of incubation at 37°C in 5% CO_2 atmosphere, an additional 0.5 ml of medium was added to each well. Twenty-four hours later, the culture media was removed, cells were extensively washed with Ca^{++}-Mg^{++}-free PBS, and 4 ml of complete medium were added to each well. Virus leftover was evaluated both in the last washing buffer and in the cell supernatants collected 3 h post-washing (time 0). Cell supernatants (500 μl) were collected at time 0 (3 h post-infection) and every 3 days post-infection to monitor p24 viral core antigen production. Fifteen days after infection, cells were harvested by trypsinization and counted by Trypan blue dye exclusion. Cell viability was above 95% for all cell types in all experiments. p24 levels in the cell supernatants was measured by an antigen capture assay (Retro-Tek HIV-1 p24 Antigen ELISA, Cellular Products Inc., Buffalo, NY).

The results showed that all three cell types were infected by HIV-1 (Figure 1A), although none of them express the CD4 molecule (Ensoli *et al*, 1995, and data not shown). However, the levels of virus production as well as the kinetics of viral replica-

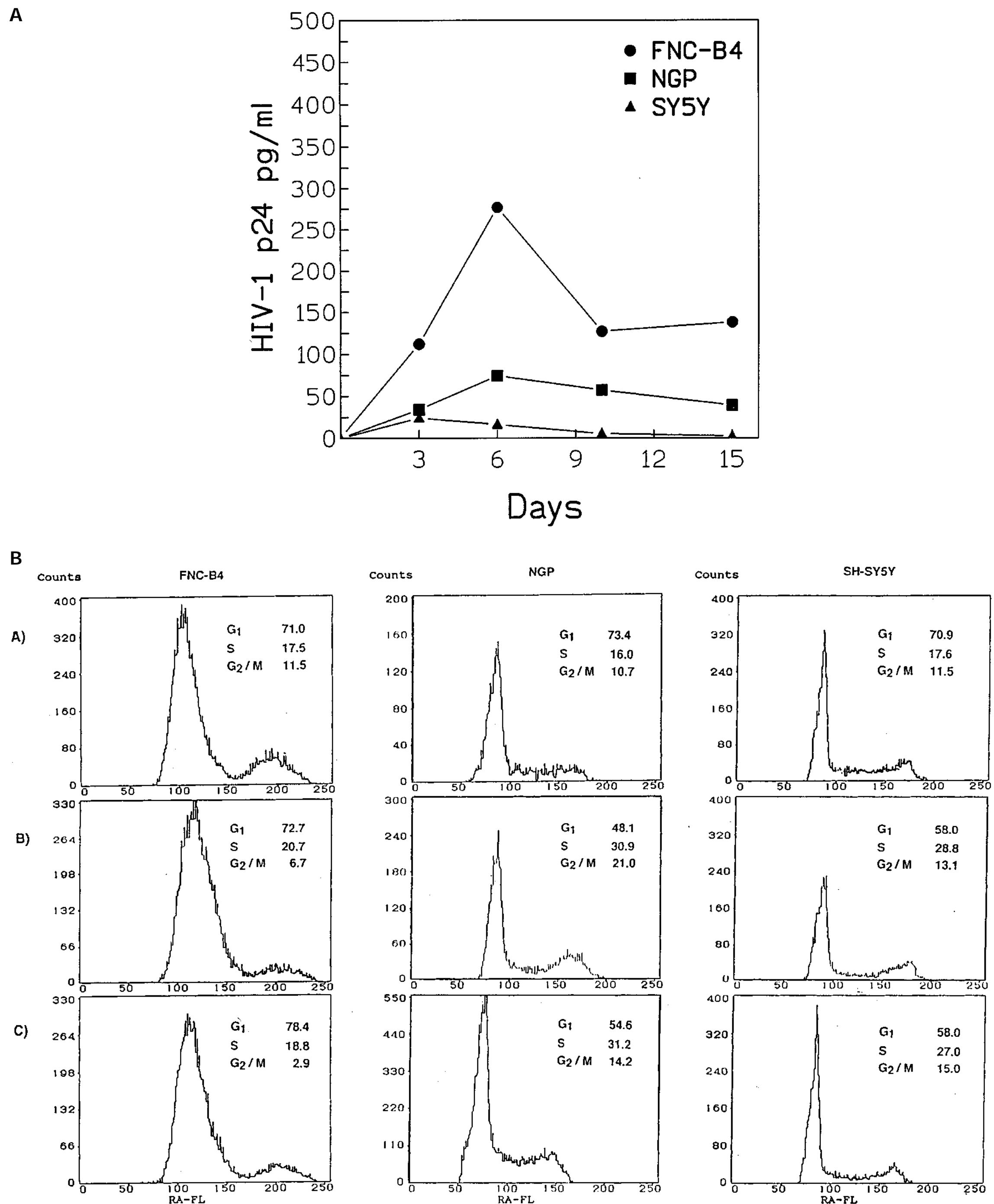

Figure 1 **(A)** HIV-1 infection and replication in embryologically and biochemically different neuroblastic cell lines. Cells were infected with equivalent titers of the monocyte-macrophagotropic (BaL) HIV-1 strain as described in the text. Viral replication was monitored by p24 viral antigen capture assay and expressed as picogram (pg) of protein per milliliter (ml) of supernatant. p24 levels at time 0 are the levels detected in supernatants collected 3 h postinfection and represent the levels of HIV-1 p24 antigen postadsorption. Each point represents the results from two to three different cultures before refeeding. The positive cutoff value of the test was calculated by adding 0.100 optical density units to the mean value of the 0 pg/ml standard (the 0 pg/ml standard was always below 0.100 optical density units or the test was considered invalid). Samples with optical density values greater than the positive cutoff value were considered to be positive. p24 concentrations as low as 5 pg/ml could be detected by antigen standards dilutions. The levels of virus replication were consistently higher with the FNC-B4 cells than NGP or SH-SY5Y cells. (**B**) Cell cycle analysis of FNC-B4, NGP and SH-SY5Y cell lines. Cells were harvested at three different time points corresponding to virus inoculum (time 0) (panel A) and days three (panel B) and six (panel C) post-infection, respectively. After staining with propidium iodide solution, cells were analysed by fluorescence-activated cell sorting (FACS) as previously described (Fiorelli *et al*, 1995).

tion differed greatly among the three cell lines, FNC-B4, SH-SY5Y, and NGP (Figure 1A). In particular, HIV-1 replication was consistently higher (3–8-fold) in FNC-B4 primary sensory neuroblasts compared to the NGP or SH-SY5Y cells which showed moderate to low levels of p24 production, respectively (Figure 1A). In addition, in the SH-SY5Y cell line HIV-1 replication progressively decreased with time (Figure 1A). This is consistent with previous studies with SH-SY5Y cell line (Ensoli *et al*, 1994a) as well as other neural cell types (i.e. immature glial cells) (Tornatore *et al*, 1994) in which very low levels of HIV-1 replication were observed. PCR or HIV-1 DNA confirmed the presence of proviral DNA, and the production of infectious virus was induced by co-culture techniques or soluble stimuli (Ensoli *et al*, 1994a; Tornatore *et al*, 1994). These results did not depend upon differences in cell viability after infection, as viability for all cell types was always above 95%; nor did they depend upon the different growth rate of these cells as indicated by the results of cell cycle analysis (Figure 1B), performed at three different time points corresponding to virus inoculum (panel A) and days three and six post-infection (panel B and C, respectively). Results of these experiments showed a comparable distribution of cycling cells (events in S and G2/M phase) with the three neuronal cell types at the time of virus inoculum (panel A). Three-days post-infection, the fraction of cycling cells progressively increases with all three cell types (panel B) and remain substantially unmodified 6 days post-infection with NGP and SH-SY5Y cells, while it decreases with FNC-B4, as these cells approach confluency (panel C). These data suggest that the lower levels of p24 production with NGP and SH-SY5Y cells, compared with those detected with FNC-B4, did not directly depend upon the distribution of cycling cells among the three distinct neuronal cell types. These results indicated that mitotically active neuroblasts consistent with sensory, sympathetic and medullary neuronal progenitors, have different susceptibilities to HIV-1 infection and suggested that HIV-1 gene expression and replication may be differentially regulated in different neuronal precursors.

HIV-1 LTR-directed gene expression in different neuronal precursors

To determine whether the differences in HIV-1 replication observed with the three neuroblastic cell lines were, at least in part, related to differing activities of the HIV-1 promoter, the cells were transfected with the wild type HIV-1 LTR-luciferase construct, and HIV-1 LTR-directed gene expression (luciferase activity) was determined after normalization to the relative transfection efficiency. In addition, we explored the activities of specific HIV-1 LTR regulatory sites in the different neuroblasts by transiently transfecting each cell line with a panel of twenty-six LTR linker-scanning mutants spanning the entire HIV-1 promoter (Figure 2). Figure 3 summarises the results of these experiments. HIV-1 LTR-directed luciferase expression was detected with all three types of neuroblasts (Figure 3), however, the levels of luciferase activity were higher (twofold) with FNC-B4 cells than NGP or SH-SY5Y cells, respectively, although transfection conditions were optimized for each cell line. As a comparison, the relatively high levels of LTR-directed gene expression observed with FNC-B4 cells were consistently low (6 –8-fold) than those detected with the CD4-positive U937 monocytic cell line, when both cell types were transfected with the same wild type LTR construct linked to the chloramphenicol acetyltransferase (CAT) reporter gene (data not shown). These data are consistent with the results of infection experiments and indicate that, although the HIV-1 LTR is functional in all three cell types, the levels of LTR-dependent HIV-1 gene expression differ among them and are lower than those observed with cells of the monocyte-macrophage lineage, which represent well known target of productive HIV-1 infection in both the developing and mature brain. Mutational analysis of viral gene expression showed additional differences among different neuroblasts such as a differing requirement and utilization of regulatory elements within the viral promoter.

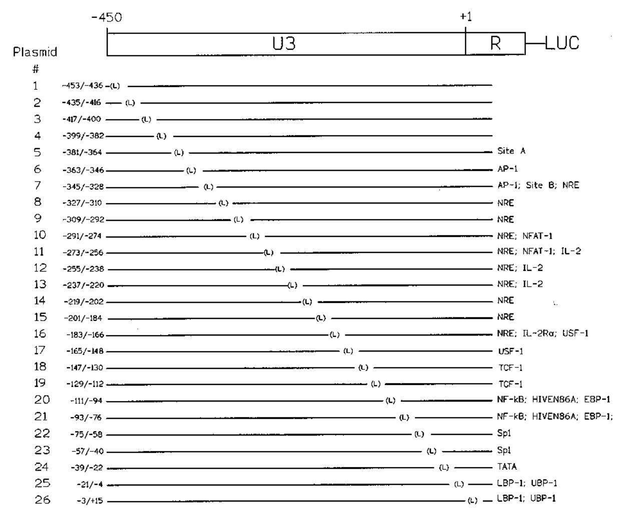

Figure 2 HIV-1 LTR linker-scanning mutants. The wild type HIV-1 LTR (−453 to +15) derived from the HXB2 clone of HIV-1, and the twenty-six linker-scanning mutants, which consecutively replace 18 bp of wild-type sequence across the U3 and R regions of the HIV-1 LTR, have been previously described (Zeichner *et al*, 1991). In the present study, the original chloramphenicol acetyl-transferase reporter gene was replaced with a luciferase reporter gene by cloning the LTR sequences into the pGL2-basic luciferase expression plasmid (Promega). Known and putative regulatory elements of the viral promoter are indicated (Antoni *et al*, 1994). LUC: luciferase gene; (L): 18 bp linker; NRE: negative regulatory elements.

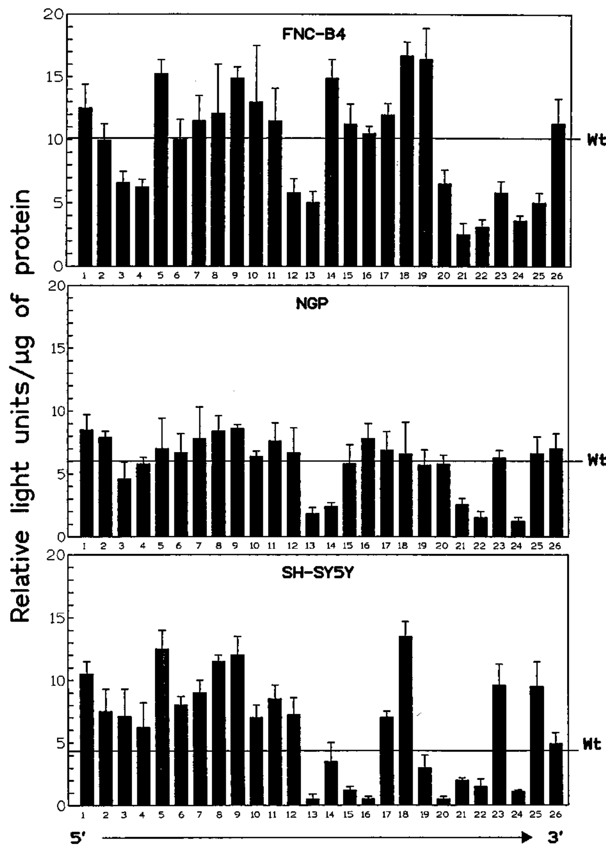

Figure 3 Activation of HIV-1 LTR-directed gene expression and mutational analysis of HIV-1 transactivation in neuroblastic cell lines with distinct biochemical and functional phenotypes. Each cell line was transiently transfected with a panel of twenty-six HIV-1 LTR Linker-scanning mutants (#1 to #26) spanning the entire HIV-1 LTR (−453/+15; figure 3). Transfections of the plasmid DNA into cells were performed by a modified lipofection procedure (Felgner *et al*, 1987), by incubating the cells with 1 ml of serum-free Coon's modified F12 medium containing 1 μg of plasmid DNA and 5 μl of lipofectamine (Life Technologies, Inc, Gaithersburg, MD) at 37°C in 5% CO_2 atmosphere. Eight hours later, 1 ml of medium containing 20% (v/v) FBS was added to each well. After an additional 12 to 48 h of incubation the medium was removed, cells were harvested by mild trypsinization, counted, and cell viability assessed by trypan blue dye exclusion. Cell viability was above 95% in all experiments. Cell extracts were prepared and used for luciferase assays as previously described (Ensoli *et al*, 1994a). The resulting light emission was measured by Luminometer (Dynatech Laboratory) and normalized to the protein content of the cell extracts. The efficiency of transfection was determined by transfecting the cells with the pCMV-βgal vector and by normalizing the levels of luciferase activity obtained with the HIV-1 LTR wild type or linker-scanning mutants to the levels obtained with pCMV-βgal after subtraction of the background luciferase activity detected with the promoter-enhancerless pGL-2 Basic plasmid (Promega). Statistical analysis was performed on the results from three or more experiments. Results were expressed as the mean ± Standard Deviation (SD) of % of Luciferase activity/100 μg of protein (relative light emission/μg of protein). Wt: luciferase activity with the wild type HIV-1 LTR. Levels of luciferase activity above or below the Wt line identify negative- or positive-acting elements, respectively.

The region of the HIV-1 LTR from nucleotides −111 to +15 (mutants #20 to #26) includes many well known regulatory sites, including the two adjacent NF-κB sites, the three adjacent Sp1 sites, the TATA element, and the binding sites for LBP-1 and UBP-1 (Figure 2). Several mutations in this region had significant effects on LTR activity in the three neuroblastic cell lines. Some of the mutations behaved consistently in all three cell lines. For example, mutant #24 (−39/−22, affecting the TATA element), mutant #22 (−75/−58, affecting the two 5′ proximal Sp1 sites), and mutant #21 (−93/−76, affecting the 3′ proximal NF-κB site), substantially decreased LTR-directed gene expression in each cell line (Figure 3). These results are consistent with previous studies with other cell types (Harrich *et al*, 1989; Jones *et al*, 1986, 1988; Lu *et al*, 1989; Nabel and Baltimore 1987; Nabel *et al*, 1988; Siekevitz *et al*, 1987), including unstimulated or functionally activated CD4-positive Jurkat cells (Zeichner *et al*, 1991), and confirm that these elements of the viral promoter are essential for efficient HIV-1 gene expression in a wide variety of cells. However, other mutants in this region behaved differently in the different cell lines. For example, the mutant #20 (−111/−94, which replaces the 5′ proximal NF-κB sites) greatly decreased HIV-1 LTR-directed gene expression only in SH-SY5Y cells, even though the 3′ proximal NF-κB site (mutant #21, −93/−76) was required for LTR-mediated gene expression in all three cell lines (Figure 3). Conversely, the levels of LTR-directed luciferase expression with mutants #23 (−57/−40), replacing the 3′-proximal Sp1, and #25 (−21/−4), affecting LBP-1 and UBP-1 binding sites, indicate that these regions of the viral promoter are required by FNC-B4 cells for wild type levels of expression, even though they do not affect HIV-1 gene expression in NGP cells and appear to exert a modest negative regulation in SH-SY5Y cells (Figure 3). These data indicate a differing requirement and utilization of NF-κB repeats and Sp1 sites among different neuronal precursors. This is consistent with previous observations with the NTERA-2 teratocarcinoma cells differentiating *in vitro* to a neuronal-like phenotype in response to retinoic acid (RA) treatment (Zeichner *et al*, 1992). The most consistent alterations of LTR transactivation among the three cell lines were observed with mutants #12 to #16, affecting the region between nucleotides −255 to −166. In this area, which includes the 3′ portion of the LTR region initially described having negative regulatory activity (the negative regulatory element or NRE) (Rosen *et al*, 1985) (Figure 2), there are distinct patterns of positive and negative regulation with the three different neuroblastic cell lines. In particular, the region identified by mutant #12 (−255/−238) was required by FNC-B4 cells for efficient LTR-directed transcription (Figure 3). However, the same motif exerted a modest negative

regulation in SH-SY5Y cells while activity in NGP cells appeared close to wild type (Figure 3. The sequences affected by mutant #13 (−237/−220) exerted a positive regulatory effect in all three cell lines, although the extent of this effect differed among the different cell lines. Mutant #14 (−219/−202), acted as a modest LTR negative-acting element in FNC-B4 cells, and as a positive regulatory element in NGP cells, while LTR activity was close to wild type in SH-SY5Y cells (Figure 3). The two mutants #15 (−201/−184) and #16 (−183/−166) define a region which appeared to have a strong positive regulation on LTR-directed gene expression in SH-SY5Y cells, however it had essentially wild type activity in both NGP and FNC-B4 cells (Figure 3). These results indicate that the region of the HIV-1 LTR comprising nucleotides −255 to −166 contains elements which are differentially active in distinct neuronal precursors. This suggests that this region of the viral promoter is important for LTR activity in immature neural cells and can be responsible for a differential regulation of HIV-1 gene expression in the developing brain. It further suggests that sequences related to those present in this region of the LTR and neuronal factors that bind those sequences may play a part in neuronal cell type-specific gene expression.

Our data indicate that different neuroblasts have distinct abilities to support HIV-1 infection and replication (Figure 1), and that this is related to both quantitative and qualitative differences in viral transactivation (Figure 3). These results suggest that neuroblast lineage-specific factors can selectively control viral gene expression and replication in different neuronal precursors, and possibly influence the degree of susceptibility of these cells to HIV-1 infection. The HIV-1 LTR includes many regulatory elements which can direct transcription in different cell types and under a variety of growth and differentiation conditions (reviewed by Antoni *et al*, 1994). Interestingly, in transgenic mice, only the HIV-1 LTR from CNS-derived HIV-1 strains were expressed in the CNS. Moreover, they were expressed almost exclusively in neurons (Corboy *et al*, 1992). This suggested that specific elements within the HIV-1 LTR can selectively control viral transactivation in developing neural cells (Corboy *et al*, 1992). In addition, a recent analysis of HIV-1 LTR sequence variation in post-mortem tissues indicated an independent, tissue-specific evolution of the viral promoter in the nervous system compared with other organ systems (Ait-Khaled *et al*, 1995). Taken together, these studies suggest that the HIV-1 LTR may play a central role in viral tissue-specific adaptation, and possibly influence viral tropism and neuropathogenicity.

Recent evidence indicate that both immature neuronal and glial cells can harbor the virus (Ensoli *et al*, 1994a, 1995; Nuovo *et al*, 1994; Tornatore *et al*, 1994; Truckenmiller *et al*, 1993). However, the extent of their involvement as well as the mechanism(s) influencing their susceptibility to HIV-1 infection and their ability to support virus replication are as yet unclear. Differentiation-dependent effects on the transcriptional activity of the HIV-1 promoter have been previously described in the embryonic carcinoma cell line NTERA-2 induced to a neuronal-like differentiation *in vitro* by retinoic acid (Zeichner *et al*, 1992). In addition, differentiation-dependent susceptibility to HIV-1 infection has been reported with both primary neurons (Ensoli *et al*, 1995; Sharpless *et al*, 1992) and tumor-derived cell lines (Ensoli *et al*, 1994a), suggesting that the susceptibility to HIV-1 infection and the control of viral gene expression and replication in neural cells may depend upon the state of cellular differentiation. These observations have important implications for pediatric HIV-1 infection, and suggest that variations in the cellular microenvironment, either lineage- or differentiation-dependent, may influence the ability of HIV-1 to infect and replicate in neural cells.

Our data indicate that the specific ability of different neuronal precursors to support viral gene expression and replication depends, at least in part, upon differing utilization of specific HIV-1 LTR regulatory elements (Figure 3). In fact, well known regulatory regions within the HIV-1 LTR such as TATA elements, Sp1 sites and NF-κB repeats, which are critically important for efficient HIV-1 transcription in a wide variety of cell types (Antoni *et al*, 1994), including CD4-positive Jurkat cells (Zeichner *et al*, 1991), are also required for maximal HIV-1 gene expression in all three neuroblastic cell lines (Figure 3). However, mutations which selectively affect the 5′-proximal or the 3′-proximal NF-κB repeats or Sp1 sites reveal a consistent variation among different neuroblasts (Figure 3), suggesting that even important regulatory elements of the viral promoter are differentially utilized by distinct neuronal precursors. Mutations clustered in the region between nucleotides −255 to −166 (Figure 3), which correspond to 3′ end of the NRE region, identified critical elements for efficient HIV-1 gene expression in these cells. In particular, alterations in this area can selectively modify the levels of LTR transactivation among the three neuroblastic cell lines. This region of the viral promoter has been shown to include cis-acting elements known to interact with factors present in different nuclear extracts (e.g. negative regulatory factor, USF-1) (Garcia *et al*, 1987; Lu *et al*, 1990; Wu *et al*, 1988). Mutations in this area can either increase or decrease HIV-1 gene expression and/or virus replication in different cell types (Lu *et al*, 1989, 1990), or in response to activation stimuli (i.e. unstimulated *versus* functionally-activated CD4-expressing lymphocytic cells) (Zeichner *et al*, 1991). Our study confirms

that this region contains both positive- and negative-acting regulatory elements, and indicates that the same element can exert either positive or negative effects on viral gene expression in different neuronal precursors, possibly through distinct recruitment of transcription factors which are involved in tissue-specific gene expression. Thus, different patterns of utilization of the same regulatory elements within the viral promoter can selectively regulate viral transactivation in different neuroblasts. This may determine preferential environments for viral replication in the developing CNS and contribute to the induction of virus latency or virus replication in the immature brain. These results also imply that strain-specific variation of specific regions of the HIV-1 LTR may influence the ability of the virus to infect and replicate in the developing nervous system, and possibly support the viral tropism toward specific neuronal precursors. This may contribute in establishing a virus reservoir in the immature CNS and participate, by either direct or indirect mechanisms, to the severity of neurological dysfunction in congenital HIV-1 infection.

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