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# Tumour necrosis factor α and interleukin-1β induce specific subunits of NFKB to bind the HIV-1 enhancer: Characterisation of transcription factors controlling human immunodeficiency virus type 1 gene expression in neural cells*

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*In human astrocytoma and neuroblastoma cell lines tumour necrosis factor α (TNFα) and* interleukin 1β (IL-1β) induced NFKB and an additional KB-specific protein of approximately 80 K to bind the HIV-1 enhancer. One nucleoprotein complex contained prototypical NFKB comprising of p50 and p65 subunits and a second contained the p65 subunit and an 80 K factor, p80. Over a 24 hr period of cytokine stimulation the p50/p65 complex of NFKB was present in the nucleus whilst the second complex declined in abundance after two hours due to the decline of p80. In unstimulated cells, DNAse I footprinting revealed a previously unidentified octamer-like binding site in the negative regulatory element (NRE) of the HIV-1 long terminal repeat (LTR) which specifically bound protein factors present in human astrocytoma, neuroblastoma and murine oligodendroglioma cell lines. A second unique motif, also in the NRE, was observed with extracts from one human neuroblastoma cell line and a murine oligodendroglioma. Footprinting analysis also confirmed that Sp1, TATA, Site A and Site B motifs of the LTR were occupied by nuclear factors present in neural cells 

Infection with HIV-1 causes a progressive syndrome of neurological disease that is not strictly dependent upon the state of immunosuppression in the infected individual (22). Although the central nervous system (CNS) is frequently the target for opportunistic infections during later stages of AIDS, the AIDS Dementia Complex (ADC) is the singular most common cause of neurological dysfunction (6). The CNS is damaged progressively leading to reactive gliosis, white matter pallor, multinucleate giant cell encephalitis or vacuolar myelopathy (4; 22) and the extent of this damage correlates with the degree of viral burden in the CNS (1). The pathogenesis of ADC is enigmatic but HIV directly infects resident neural cells (3; 10; 27) in addition to the more prevalent infection of infiltrating or resident monocytic cells that follows initial infection and probably mediates the viral invasion of the CNS (27). Infection of the brain is more frequently detected in astrocytes than neuronal or oligodendroglial cells and in astrocytes *in vitro* HIV exhibits characteristics of a latent infection with little then no virus production (30). Recent analysis of brain tissues from cases of paediatric and adult AIDS demonstrates that HIV undergoes a similar restricted pattern of gene expression in astrocytes *in vivo* which is marked by the absence of structural mRNAs and the predominant expression of the *nef* gene product (3). Screening sections of such post-mortem tissue by immunocytochemistry and *in situ* hybridisation with Nef-specific probes indicates that as many as 20% of astrocytes present in regions of reactive gliosis are infected (3).

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The mechanisms by which the reactivation of HIV replication occurs in the CNS are therefore of considerable importance as neural cell infection represents a much larger viral reservoir than suggested previously (3). In lymphocytic and myeloid cells the cytokine TNFα is a strong activator of HIV-1 replication (23) and greatly increases transcription from the viral LTR through the induction of proteins from the NFκB/c-*rel* family of transcription factors that bind to the HIV-1 enhancer (17, 20). Parallel studies in neural cells demonstrate that both TNFα and IL-1β are capable of augmenting gene expression from the HIV-1 LTR in neuronal and astroglial cell types (28) and will activate virus production from latently infected astrocytes *in vitro* (30). TNFα also augments HIV-1 replication in several neuronal cell lines and IL-6 has a similar effect in one of them (32). The enhancement of HIV-1 LTR-driven gene expression by TNFα and IL-1β further correlates with the specific induction of an NFκB-like activity that binds the enhancer (28). Here we report the characterisation of the cellular proteins that constitute this cytokine-inducible DNA-binding activity in astrocytoma and neuroblastoma cells and the identification of binding sites for transcription factors which interact with other regions of the HIV-1 LTR in these and other neural cell lines.

## METHODS

*Cell lines, cytokines and antisera* The cell lines and cytokines employed have been described previously (28). Polyclonal antisera to human NFκB subunit p50 and subunit p65 were kindly donated by Dr. R. T. Hay, University of St. Andrews, St. Andrews, Fife UK.
*Electrophoretic Mobility Shift Assays (EMSA)* The preparation of nuclear extracts and EMSA have been described previously (28). A double-stranded oligonucleotide containing a single NFκB motif complimentary to the HIV-1 enhancer was employed in EMSA. The protein binding site is underlined. For UV cross-linking the probe contained 5'-bromodeoxyuridine (BUdR) substitutions at the positions indicated by bold text.

5'-TGAGGGACTTTCCGC-3'
3'-ACTCCCTGAAAGGCG-5'

*UV cross-linking* Preparative-scale gel retardation assays were performed with a BUdR substituted NFκB oligonucleotide probe, wet gels were exposed to UV light (UV Products Inc. transilluminator) for 30 min at 4°C and autoradiographed for 16 hrs. Specific protein-DNA complexes were excised, the gel slice crushed and the proteins extracted by diffusion into buffer (100 mM Tris-Cl [pH 7.4], 2% SDS, 1 mM DTT) for 4 hrs on a rotary wheel. Polypeptides were recovered by precipitation with 2 vol cold acetone and resolved on 7.5% SDS polyacrylamide gels. Following electrophoresis gels were fixed, subject to fluorography, dried and autoradiographed for 4 days at -70°C.
*DNAse I footprinting* DNA fragments derived from the HIV-1 LTR (LAI isolate) were radiolabelled by standard techniques at one terminus for each strand and a limited DNAse I digestion of 2-5 femtomoles of LTR probe was performed with nuclear extract (0-200μg) in the presence of 4μg poly [dI:dC]-[dI:dC] (9). Reaction products were separated on 8% denaturing acrylamide gels with parallel electrophoresis of Maxam and Gilbert DNA sequencing reactions (2) of the corresponding LTR probe. Gels were fixed, dried and autoradiographed at -70°C for 6 days.

## RESULTS

UV-crosslinking analysis of the HIV enhancer-specific complexes, formed in EMSA by nuclear extracts from TNFα-treated SK-N-MC neuroblastoma cells and IL-1β-treated U373MG astrocytoma cells, to a BUdR substituted κB probe allowed estimation of the molecular weights of the protein factors involved (Fig. 1A). Proteins cross-linked to the κB probe resolved into three polypeptides of approximately 53 K, 68 K and 80 K on SDS-polyacrylamide gels which suggested that they may represent known members of the NFκB/c-*rel* family of transcription factors; p49 or p50, p65 and c-*rel* [p85] (17), particularly as the cross-linking of a such a small (15 bp) oligonucleotide probe does not discernibly alter the estimation of molecular weight (16). No nuclear factors capable of binding to the tandem NFKB recognition sites in the HIV-1 LTR were present in neuroblastoma and astrocytoma cells prior to exposure to these cytokines (28, 29) and the specificity of binding has been confirmed previously by competition with mutant and wild type NFKB probes in EMSA (29).

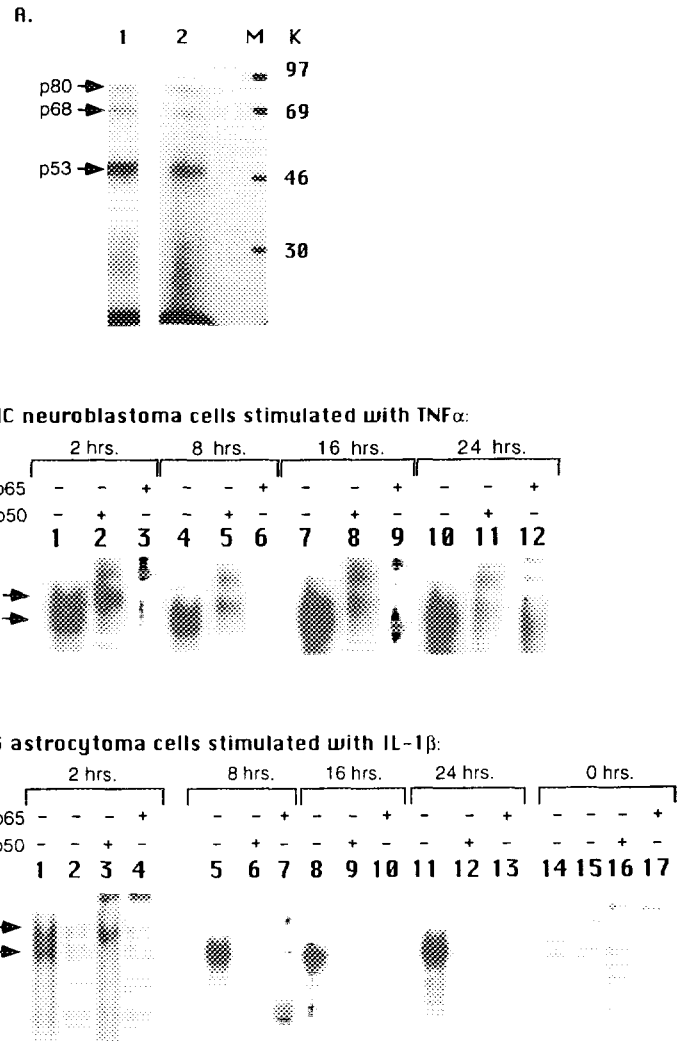


Fig.1. *KB-specific enhancer-binding proteins in SK-N-MC neuroblastoma and U373MG astrocytoma cells after stimulation with TNFα and IL-1β.* A) UV-crosslinking of specific KB complexes after EMSA. Lane 1), SK-N-MC stimulated with 100 U/ml TNFα for 2 hrs; 2), U373MG stimulated with 250 U/ml IL-1β for 2 hrs. M=protein molecular weight standards. B) and C), Inhibition of binding to a KB oligonucleotide probe in EMSA by antisera to NFKB subunit p50 or p65. B), SK-N-MC stimulated TNFα and C), U373MG stimulated IL-1β for 2 to 24 hrs. Lanes 2 and 15 show competition with a 50 fold molar excess of unlabelled KB probe prior to assay.

The NFκB binding activity was maintained in the nucleus of these cells over a period of 2 to 24 hrs of cytokine stimulation (Fig. 1B and C) and in EMSA's two distinct complexes were observed after 2 hrs of cytokine stimulation yet at later times only one complex could be discerned clearly. The cellular components of the nucleoprotein complexes were identified with antisera to the NFκB subunits p50 and p65. In nuclear extract from neuroblastoma cells stimulated with 100 U/ml TNFα for 2 hrs antibody to p50 abolished the formation of the lower complex [LC] but not the upper complex [UC] (Fig. 1B). After 8, 16 and 24 hrs of TNFα stimulation p50 antibody inhibited the formation of the single nucleoprotein complex, equivalent to LC in mobility, and in addition to a super-shifted complex (17b) promoted the formation of the upper complex which was not normally present at later times. A similar pattern of NFκB complex formation was observed in nuclear extracts

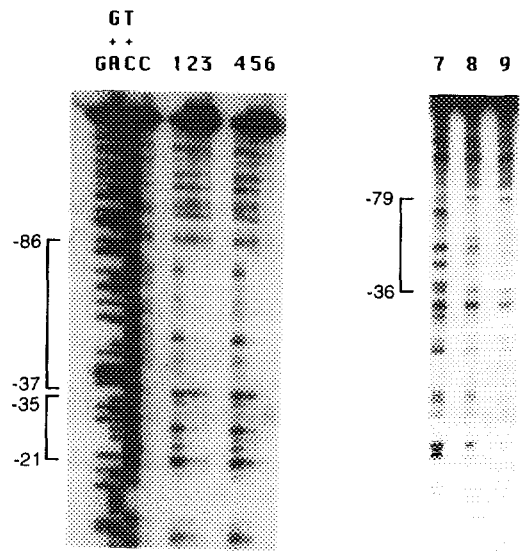


Fig. 2. *DNAse I footprinting of the -158 to +78 probe* (LTRF) labelled on the non-coding strand, with nuclear extracts from unstimulated G26-24 oligodendroglioma, U373MG astrocytoma cells and labelled on the coding strand, with nuclear extracts from SK-N-MC neuroblastoma cells. G, G+A, T+C, C denote Maxam and Gilbert sequencing reactions. Lanes 1, 4 and 7, no protein; 2 and 3, G26-24 100μg and 200μg; 5 and 6, U373MG 100μg and 200μg; 8 and 9, SK-N-MC 75μg and 150μg.

from astrocytoma cells following stimulation with 250 U/ml IL-1β (Fig. 1C). After 2 hrs of IL-1β stimulation p50 antisera abolished the formation of LC. However, in these cells at longer periods of IL-1β treatment the inclusion of anti-p50 did not lead to the appearance of the upper complex. An antiserum specific for the 65 K subunit of NFκB, p65, abolished or severely reduced the formation of both LC and UC with nuclear extracts from neuroblastoma and astrocytoma cells after short periods (2hrs) of cytokine stimulation and similarly inhibited the formation of UC at the later time points (Fig. 1B and C). Although this is less clearly shown in SK N MC at 16 and 24 hours of TNFα stimulation due to degradation of the proteins binding to the NFKB probe. The anti-p65 preparation also frequently produced an additional band to that observed in control reactions due to the ability of this antisera to super-shift specific nucleoprotein complexes (17b). From these experiments our data indicates that the p50 and p65 components of NFκB constitute one nucleoprotein complex [LC] in EMSA whilst the second complex [UC], present only after initial cytokine stimulation, is comprised of p65 but not p50. Given the universal finding that members of the NFκB/*c-rel* family bind to DNA as dimers (17) results from UV-crosslinking and EMSA suggests that p65 forms a complex with the 80 K protein.

DNAse I footprinting analysis of nuclear extracts from U373MG astrocytoma, SK-N-MC and SK-N-SH neuroblastoma cells, and some assays with a murine oligodendroglioma cell line G26-24, were performed to identify the DNA-binding activities that recognised the HIV-1 LTR in unstimulated neural cells. The HIV-1 LTR was cleaved with restriction enzymes to generate three probes suitable for footprint analysis which spanned the LTR from nucleotides -488 to -306 (Probe 194), -305 to -159 (Probe 159) and -158 to +78 (Probe LTRF) relative to the start of transcription at +1. Only data relating to the DNA-binding factors present in extracts from cells not stimulated with cytokines is presented as no differences were observed in the pattern of DNA-binding factors other than the induction of NFκB by TNFα or IL-1β in footprinting or EMSA analyses employing the same radiolabelled DNA probes or various complimentary oligonucleotide probes (29).

The DNA sequences in probe LTRF include the tandem NFκB sites of the enhancer, the three Sp1 sites, TATA box and TAR regions of the LTR (12). After binding of this probe to nuclear

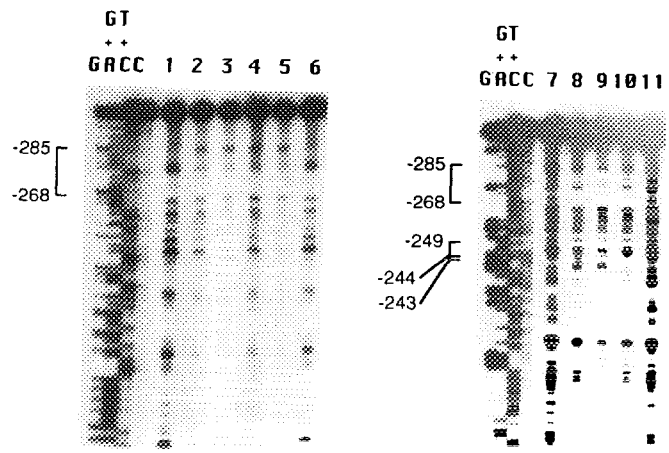


Fig. 3. *DNAse I footprinting of the -305 to -159 probe* (159) labelled on the coding strand, with nuclear extracts from U373MG astrocytoma and SK-N-SH neuroblastoma cells and labelled on the non-coding strand, with nuclear extracts from G26-24 oligodendroglioma cells and SK-N-MC neuroblastoma cells. G, G+A, T+C, C denote Maxam and Gilbert sequencing reactions. Lanes 1, 6, 7, and 11, no protein; 2 and 3, U373MG 75μg and 150μg; 4 and 5, SK-N-SH 75μg and 150μg; 8 and 9, G26-24 75μg and 150μg; 10, SK-N-MC 100μg.

extracts from astrocytoma or oligodendroglioma cells footprinting identified a region of strong protection from DNAse I cleavage over all three Sp1 sites from -86 to -37 and partial protection over the TATA box from -35 to -21 (Fig. 2). Sequences further downstream could not be resolved due to the proximity of the radiolabel relative to the potential protein binding sites defined previously in the TAR region (12). In SK-N-MC neuroblastoma cells footprinting confirmed the binding of proteins over the Sp1 sites (Fig. 2). Protein binding to an Sp1 consensus oligonucleotide probe was also observed in EMSA with nuclear extracts from astrocytoma, and both neuroblastoma cell lines (29). Probe 159 covered the 3' region of the NRE in the LTR and sequences upstream of the enhancer which contain binding sites for USF (8) and the NFAT-1 (26) and TCF-1α proteins (12) present in activated T cells. Here footprinting analysis demonstrated protection over a previously undefined region from -285 to -268 in astrocytoma, both neuroblastoma and the oligodendroglioma cell lines (Fig. 3). Although the exact 3' boundary of this site may not have been defined accurately due to this sequence containing a region rich in adenosine residues that is a poor substrate for DNAse I (9), the 5' border was defined by a protein-induced DNAse I stop at -285. The protected sequence centred on 8 bp, 'AGGCCAAT' which differs from the consensus octamer-binding motif 'ATGCCAAT' by only one nucleotide (14). A second novel footprint was also observed with extracts from SK-N-MC neuroblastoma cells from -249 to -243 and in oligodendroglioma cells from -249 to -244 over the sequences 'ATGTGGG' and 'ATGTGG', respectively. This sequence is similar to a region of the SV40 promoter known as the GTI motif where 'GGTGTGGG' was defined as a recognition sequence that interacts with several ubiquitous cellular proteins (14). The 5' terminal portion of the LTR, probe 194, contained the 5' region of the NRE and Site A and Site B binding sites defined previously in a T cell line (19). DNAse I footprinting of this probe demonstrated protection from cleavage over Site A and Site B in astrocytoma and both neuroblastoma cell lines (Fig. 4). The Site A sequence showed a slightly smaller footprint than in the T cell line Jurkat whilst protein binding over the Site B region in neural cell extracts was evident by two regions of protection from -355 to -346 and -337 to -326 separated by a site of strong DNAse I sensitivity which together defined a similar region containing the two palindromic half sites characteristic of a steroid hormone-receptor motif (19). Subsequently the Site B binding activity in the T cell line was identified by others (5) as the human relative of COUP-TF, the chicken ovalbumin upstream promoter transcription factor, a member of the

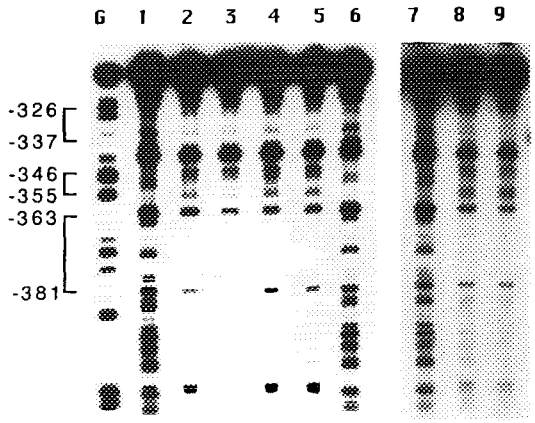


Fig. 4. *DNAse I footprinting of the -488 to -306 probe* (194) labelled on the coding strand with nuclear extracts from SK-N-SH and SK-N-MC neuroblastoma and U373MG astrocytoma cells. G denotes Maxam and Gilbert sequencing reaction. Lanes 1, 6 and 7, no protein; 2 and 3, SK-N-SH 75μg and 150μg; 4 and 5, SK-N-MC 75μg and 150μg; 8 and 9, U373MG 75μg and 150μg.

steroid/thyroid hormone receptor super-family. Further analysis of Site B binding activity from astrocytoma and SK-N-SH neuroblastoma cells in EMSA using probe 194 and competition with Site B oligonucleotides containing one or both half sites demonstrated that both half sites were recognised and required for maximal binding activity (29). Therefore the production of two regions of protection in footprinting assays most probably represented a product of partial protection of the binding site.

## DISCUSSION

It has been established in T-lymphocytes that the induction of NFκB has an important role in the regulation of HIV replication where it can be induced by the cytokine TNFα (20) and during cellular activation following antigenic stimulation of the T-cell receptor (17). The NFκB motifs in the HIV-1 LTR interact with at least four members of the NFκB/c-*rel* family of transcription factors which form heterodimers in combinations in order to specifically bind DNA. In addition to the p50/p65 (NFκB-1) and p49/p65 (NFκB-2) interactions another member, c-*rel* [p85] will form heterodimers with p50 or, less frequently, p65 (17). The p65 subunit of NFκB contains a strong transcriptional activation domain, in addition to DNA-binding and dimerization regions also present in p50 (24) and defines a mechanism whereby TNFα, through NFκB binding, augments gene expression from the HIV-1 LTR and hence viral replication (20, 23). In neural cells both TNFα and IL-1β augment gene expression from the viral LTR (28) through an analogous mechanism of NFκB induction involving the binding of prototypical p50/p65 NFκB-2 and p65 in conjunction with another factor p80 to the HIV-1 enhancer. Viral replication is similarly initiated by TNFα and IL-1β in primary human astrocytes latently infected with HIV-1 (30) and demonstrates that these cytokines acting through the processes described here have the potential to activate virus expression in the reservoir of infected astrocytes *in vivo*.

DNAse I footprinting analysis of the HIV-1 LTR demonstrated the interaction of nuclear factors with an octamer-like motif located in the NRE in all neural cell types and with the GTI-r motif, also in the NRE, in the SK-N-MC neuroblastoma and murine oligodendroglioma cells. The

interaction of a nuclear factor(s) with these motifs in the LTR has not been previously described and may occur specifically in neural cells. Octamer-binding factors have been identified which participate in antigen-specific activation in T lymphocytes (31) and cell-specific transcription of the immunoglobulin promoters in B lymphocytes (14), and several other factors, as well as many that recognise simply the 'CCAAT' sequence, appear ubiquitous (14). A number of octamer-binding proteins, including one identical to the lymphoid specific activator Oct-2, have also been identified in brain extracts and a glioma cell line (13). This 8 bp motif partially overlaps a purine-rich domain recognised by the cellular factor ILF present in lymphoid and HeLa cell lines (15) and competition may account for this activity not having been reported previously to bind the HIV-1 LTR. The octamer-like motif is 100% conserved in 14/19 cloned HIV-1 isolates and the GTI-r motif is similarly 100% conserved in 17/19 of these isolates (18). The finding of unique protein-binding sites in the LTR may have specific implications for the regulation of HIV-1 gene expression in neural cells and since binding to these sites was not influenced by stimulation with TNFα or IL-1β (29) they must operate in mechanisms that regulate other aspects of transcription. Although binding to the GTI-r site may augment basal expression from the LTR as the SV40 GTI motif has been shown to bind an Sp1-like factor (14) a constitutive octamer factor (Oct-1) in conjunction with an inducible factor ($OAP^{40}$) contributes to the induction of IL-2 gene expression following T cell activation (31) and allows speculation that the octamer-like motif may regulate transcription in an inducible manner to other cellular stimuli in neural cells, for example those provided by neuro-active peptides. By analogy the interaction of protein factors present in neural cells to the previously recognised Sp1, TATA, Site A and Site B binding sites in the HIV-1 LTR may parallel the data on their function in T lymphocytes. In these cells the interaction of Sp1 to the three sites downstream of the enhancer is required for basal expression from the LTR and the Sp1 motifs are necessary for optimal transactivation by Tat (21). Although the function of Site A is unknown, the COUP-TF homologue, a member of the steroid hormone superfamily that recognises Site B (5) may have a negative influence on HIV-1 expression as the mutation of Site B increases gene expression from an LTR-driven reporter construct (19). In contrast, the chicken factor from which the human Site B binding activity was identified (5) activates transcription, in conjunction with a second protein S300-II, and represents a class of steroid hormone receptors where either no ligand is required for DNA binding or an additional protein(s) replaces the normal requirement (33).

The conservation in detail of HIV regulation between neural and lymphoid cells observed is not unexpected and will be beneficial to the preparation and application of anti-HIV therapies to AIDS dementia, such as those designed to limit the systemic or intrathecal production of TNF or reduce the cellular redox balance and hence limit the activation of NFκB (25). However, the finding of neural-specific factors that interact with the transcriptional machinery of HIV may provide further opportunities for interference in the virus life cycle within such infected cells present in the central nervous system.

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