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HIV-1 Tat directly binds to NFκB enhancer sequence: role in viral and cellular gene expression

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ABSTRACT

HIV-1 Tat protein reprograms cellular gene expression of infected as well as uninfected cells apart from its primary function of transactivating HIV-1 long terminal repeat (LTR) promoter by binding to a nascent RNA stem–loop structure known as the transactivator response region (TAR). Tat also induces chromatin remodeling of proviral LTR-mediated gene expression by recruiting histone acetyl transferases to the chromatin, which results in histone acetylation. Furthermore several studies have shown convincing evidence that Tat can transactivate HIV-1 gene expression in the absence of TAR, the molecular mechanism of which remains to be elucidated. Here we show a direct interaction of Tat with nuclear factor kappa B (NFκB) enhancer, a global regulatory sequence for many cellular genes both *in vitro* and *in vivo*. This interaction not only provides a novel molecular basis to explain TAR-independent transactivation in HIV-1, but also points toward the potential mechanism of Tat-mediated modulation of cellular genes.

INTRODUCTION

The transactivator protein Tat of human immunodeficiency virus-1 (HIV-1) is the most important regulator of viral gene expression and replication (1,2). Tat has been shown to activate long terminal repeat (LTR) promoter directed transcription by interacting with nascent RNA stem–loop structures, present immediately downstream of the initiation sequence known as the transactivation response region (TAR) (3,4), and subsequent assembly of positive transcription elongation factor b (pTEFb) (5). This activity is dependent on various cellular (6) and viral factors (7) including cyclin T1 and Tat-activated kinase (TAK) (8), which forms a ternary complex via metal ion coordination (9). Tat has been shown to be involved in both initiation and elongation of transcription (2,3,10). It is also known to induce chromatin remodeling (11) by recruiting histone acetyl transferases (HATs) such as p300 and p300/CBP associated protein P/CAF to the chromatin (11–13), which results in histone acetylation (14) and acetylation of itself (15). In addition, several studies have shown convincing evidence that Tat can transactivate HIV-1 gene expression in the absence of TAR (16–18). It has also been shown that NFκB regulatory elements in the enhancer region of LTR play an important role in TAR-independent transactivation but the molecular basis of this activation remains to be clearly understood (18,19). Furthermore, an absolute requirement of NFκB enhancer element was shown for both Tat-dependent and Tat-independent viral transcription in blood CD4 T cells (20). TAR-independent transactivation of LTR by Tat illuminates the complexity underlying the modulation of viral and cellular promoters, suggestive of potential pathways responsible for its multiple functions.

The overview of the molecular activities of Tat clearly indicates that, far beyond an HIV-1 specific transcriptional transactivator, the protein acts as a pleiotropic factor for a number of functions both inside and outside the cell (6). Tat protein through transcellular communication reprograms cellular gene expression of infected as well as uninfected cells and may contribute to a wide range of clinical complications (21). It has been shown to modulate a number of cellular genes and make the cellular environment amenable for viral replication (6). A number of earlier studies clearly indicate that Tat could substantially affect transcription when tethered to DNA (18,22). There have also been reports establishing functional similarities between Tat and other transcription factors (23) which enhance the level of gene expression by binding to DNA. It is also established that Tat exist as a metal ion (Zn^{2+}or Cd^{2+})-linked dimer bridging cysteine-rich regions of each monomer, a characteristic of DNA binding proteins (24). In addition, Tat is shown to modulate and de-repress the integrated HIV-1 chromatin structure, aiding in activation of transcription; however, NFκB alone fails to stimulate the integrated transcriptionally silent HIV-1 promoter (25,26). Interestingly, Tat is unable either to activate transcription or to induce changes in the chromatin structure of an integrated promoter lacking both Sp1 and NFκB sites even when it is tethered to the HIV-1 core promoter upstream of the TATA box (25), indicating thereby the importance of this region in LTR driven viral gene expression in infected cells.

All this information leads us to ask whether Tat could interact with DNA, specifically with upstream enhancer sequences in LTR, which has been shown to be important for both TAR-independent and TAR-dependent Tat-responsive transactivation of HIV-1 LTR. In this report we

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demonstrate for the first time a direct interaction of Tat both *in vitro* and *in vivo* with NFκB enhancer element, which has previously been suspected to be a critical element for TAR-independent transactivation by Tat.

MATERIALS AND METHODS

Oligonucleotides and Tat protein

Consensus NFκB, AP1 and SP1 oligonucleotides were obtained from Promega (USA). All other oligonucleotides were custom synthesized using β-cynoethyl phosphoramidite chemistry on either Pharmacia Gene Assembler plus automated synthesizer (Pharmacia, USA) or ABI 3900 DNA synthesizer (Applied Biosystems, USA). Oligonucleotides were purified to more than 95% purity on a C18 reverse phase HPLC column using triethyl ammonium acetate buffer.

Recombinant pure Tat was obtained from the NIH AIDS Reagent Program (27) or purified from *Escherichia coli* BL21-DE3 transformed with expression vector GST-Tat 1-86R TK and GST-Tat 1-86 C22G mutant (28) as reported, with minor modifications. The GST-Tat bound glutathione–Sepharose beads (Amersham, USA) were treated with thrombin (Amersham, USA) in PBS pH 7.4 to cleave the fusion tag. The protein was further purified by reverse phase FPLC on a C4 column with water:acetonitrile and 0.1 % trifluroacetic acid buffer, and purity was checked by C18 reverse phase HPLC and SDS–PAGE followed by silver staining and western blot analysis. The polyclonal Tat antibody (29) that has been reported to work well in both immunoprecipitation and western blot (7) was obtained from the NIH AIDS Reagent Program.

Cell cultures and preparation of nuclear extract

HEK 293T and Jurkat (J6) T-cell lines were obtained from the Cell Repository, National Centre for Cell Science, India. CEM-GFP, a reporter T-cell line, was obtained from the NIH AIDS Repository (30). HEK 293T cells were grown in Dulbecco's modified Eagle's medium (Gibco-BRL, USA), CEM-GFP cells and Jurkat cells were grown in RPMI 1640 (Gibco-BRL, USA) supplemented with 10% fetal bovine serum (Gibco-BRL, USA) at 37°C in 5% CO_2. The nuclear extract of Jurkat cells activated with 50 ng/ml of phorbol 12-myristate 13-acetate (PMA) (Sigma, USA) or untreated cells was prepared essentially as described earlier (31).

HIV-1 infection and transfection

The CEM-GFP cell line was infected with HIV-1 NL4.3 virus isolate (32) using 100 ng of p24 units per 2×10^6 cells as described previously (33). A reporter vector expressing luciferase under five tandem copies of NFκB enhancer, pNFκB-Luc, was obtained from Stratagene (USA). The HIV-1 Tat encoding expression vector pCDNA-Tat, has been described previously (7). The 1–48 truncated Tat gene was cloned in pCDNA3.1 by subcloning the sequence from the pCDNA-Tat vector described above using PCR. Jurkat cells were stably transfected with pCDNA-Tat using electroporation and the cells were incubated with geneticin (G418 sulfate; Gibco-BRL, USA) 1000 μg/ml for selecting Tat-expressing Jurkat cells. Reverse transcription PCR and transactivation assay using HIV-1 LTR-luciferase vector confirmed the expression of Tat in these Jurkat-Tat cells. Nuclear extracts from Jurkat-Tat cells were prepared as mentioned above. HEK 293T cells were plated at a density of 6×10^5 cells/well in a six-well plate. The cells were allowed to adhere to the plate and subsequently plasmid vectors were transfected with Lipofectamine 2000 (Gibco-BRL, USA) according to the manufacturer's instructions.

Gel shift assay

Complementary strands of oligonucleotides were annealed to generate double-stranded oligonucleotides for gel shift assay by heating equimolar amounts at 94°C for 2 min and subsequently gradually cooling to room temperature in a water bath (Table 1). Oligonucleotides were end-labeled with [γ-^{33}P]ATP (BRIT, India) and 10 U of T4 polynucleotide kinase (Gibco-BRL, USA) using a forward reaction buffer according to the manufacturer's instruction. Radiolabeled probes (0.02 pmol) were incubated with 2.5 μg of pure Tat protein, unless indicated otherwise, in 10 mM HEPES pH 7.9, 50 mM KCl, 1 mM dithiothreitol (DTT), 2 μg/ml poly dI-dC:dI-dC, 330 μg/ml BSA and 10% (w/v) glycerol at 30°C for 10 min and loaded onto 9% native PAGE (acrylamide:bisacrylamide, 50:1) containing 5% glycerol. The gel was electrophoresed in 0.5× TBE at 150 V for 45 min at 4°C in a Bio-Rad protean II gel electrophoresis system. The gel was dried and exposed to Kodak Biomax film with intensifying screen. A competition assay was carried out using 100- and 200-fold molar excess of cold specific or mutated oligonucleotide. Supershift was done using anti-Tat antibody (29) and anti-p65 antibody (Santa Cruz, USA).

Oligo library screening

The synthetic random oligonucleotide library (DDSEL) with two fixed primer regions (5′ DDCI and 3′ DDCII) and a central random 32-base region was synthesized using the reported procedure (34). The oligonucleotide library was desalted and used without any further purification. One hundred nanograms of DDSEL was labeled with [α-^{32}P]dATP (BRIT, India) using an *E.coli* Klenow fragment (Roche, USA) according to the manufacturer's instruction. The selection of bound oligonu-

Table 1. List of oligonucleotides used in gel shift assay

Sr. no.	Sequence	Region (position)
1	5′ CAAGGGACTTTCCGCTGGGGACTTTCCAGG	NFκB enhancer from HIV-1 LTR (347–376)
2	5′ CAACTCGGTTTCCGCTCTCAGCTTTCCAGG	Mutated NFκB enhancer from HIV-1 LTR
3	5′ AGTTGAGGGGACTTTCCCAGGC	NFκB consensus
4	5′ AGTTGACTCTCAGATGATAGGC	Mutated NFκB consensus
5	5′ ATTCGATCGGGGCGGGGCGAGC	SP1 consensus
6	5′ CGCTTGATGAGTCAGCCGGAA	AP1 consensus

Table 2. List of oligonucleotide primers used in CHIP assay

Sr. no.	Sequence	Region (position)
1	5′ CCTGCATGGAATGGATGACC	HIV-1 LTR forward (218–237)
2	5′ CGCCCAGGCACGCTCC	HIV-1 LTR reverse (376–393)
3	5′ CGAACAGGGACTTGAAAGC	HIV-1 LTR forward (643–661)
4	5′ CATCTCTCTCCTTCTAGCCTC	HIV-1 LTR reverse (772–792)

cleotides was done after incubation with GST-Tat protein followed by gel shift as described above. The bound oligonucleotides were gel extracted using the crush and soak method. The gel-extracted band was radiolabeled during amplification by PCR using DDCI and DDCII primers in the presence of [α-^{32}P]dATP. This product was used for the next round of selection following the same protocol. Five rounds of iterative selection were carried out by gel shift and the enriched library was cloned into pGEM-T Easy vector (Promega, USA) according to the manufacturer's instructions and transformed into chemically competent *E.coli* DH5α. Fourteen positive clones were selected and sequenced.

Circular dichroism (CD)

CD spectra were collected on a Jasco-715 spectropolarimeter using a 1 nm bandwidth. CD spectra were averaged for 10 accumulations at a scan speed of 100 nm/min to improve the signal-to-noise ratio. A quartz cell with a pathlength of 0.10 cm was used for all measurements. All spectra were collected by dilution of the protein and oligonucleotides in PBS pH 7.4.

Chromatin immunoprecipitation (CHIP) assay

The CHIP assay was done as described (35,36) with minor modifications. Briefly, HIV-1 NL4.3-infected CEM-GFP cells were cross-linked by 1% formaldehyde followed by quenching with 125 mM glycine. Cells were washed and the pellet was lysed in lysis buffer [150 mM NaCl, 25 mM Tris–HCl pH 7.5, 5 mM EDTA pH 8.0, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM PMSF, 10 mM sodium butyrate, 1× protease inhibitor cocktail (Roche)], followed by sonication. The sonicated lysate was precleared by incubation with protein A/G beads, salmon sperm DNA and BSA followed by centrifugation. This supernatant was used as the input sample for immunoprecipitation with anti-Tat antibody or isotype control by incubation at 4°C overnight. The chromatin antibody complex was immobilized on protein A/G beads and then eluted in 2% SDS, 0.1 M $NaHCO_3$ and 10 mM DTT. Cross-links were reversed and the protein was digested with proteinase K (100 μg/ml). DNA was recovered by phenol–chloroform extraction and ethanol precipitation. Precipitated DNA was dissolved in water and analyzed by PCR (30 cycles: 94°C for 1 min, 55°C for 1 min, 72°C for 1 min) with the LTR specific primers described in Table 2. The PCR products were analyzed on 1% agarose gel and visualized by ethidium bromide staining.

RESULTS

Although the previous reports point toward the possibility of a DNA binding activity of Tat (18,22,23), to the best of our knowledge it has not been shown experimentally. In order to identify the presence of DNA binding activity in Tat protein, we started with an alignment search for Tat sequence against superfamilies of protein, based on the structural classification of proteins using the hidden Markov model (37) with software Superfamily 1.61 (http://www.supfam.org). This examination of HIV-1 Tat protein suggested that it contained structural motifs homologous to the N-terminal domain of the mouse and human NFκB p50 subunit (Fig. 1) and that it belongs to the p53 superfamily of transcription factors. This similarity in structural motif could be due to the presence of structurally conserved amino acid residues like Val (4th), Cys (22nd) Leu-Gly-Iso (43rd to 45th) and Lys-Lys (50th to 51st).

Figure 1. Multiple alignment of sequence obtained from SCOP database searches with Superfamily version 1.61. Tat protein aligns with a p53-like transcription factor superfamily with maximal homology to the N-terminal domain of mouse and human NFκB p50 (model number 0003904; expectation value, $2.9 \times 10^{-0.1}$). The proteins are identified by PDB entry codes on the left-hand side of the alignment. The following proteins show alignment: mouse NFκB p50 dimer (1nfk), human NFκB p50 dimer (1svc), chicken c-Rel dimer (1gji), mouse NFκB p65 dimer (2ram), mouse NFκB p65 p50 human IκB complex (1ikn), human NFκB p65 p50 human IκB complex (1nfi), mouse NFκB p65 p50 dimer (1vkx) and *Anopheles* gambif1 dimer (1bvo).

Based on previous reports and our structural alignment results mentioned above, we used gel shift assay to identify the DNA binding activity of purified recombinant Tat protein with NFκB enhancer sequences. As shown in Figure 2A, Tat protein specifically binds to NFκB consensus sequence, which is competed out by cold specific oligo but not by mutated oligo. Tat binding to the DNA was further confirmed by a supershift using Tat antibody (Fig. 2A, lane 6). Then we used the oligonucleotide sequence present in the HIV-1 LTR representing the NFκB enhancer sequence (Table 1) for Tat interaction in gel shift assays. Again, Tat specifically bound the wild-type LTR NFκB while no binding was observed with a mutated oligo (Fig. 2B). To establish the specificity of this binding, we performed gel shift assay with two additional enhancer oligonucleotides present in the HIV-1 LTR, AP1 and SP1. Neither of these sequences showed any interaction with Tat protein although they interacted with the cellular proteins present in nuclear extract (Fig. 2C), demonstrating thereby the specificity of Tat-NFκB enhancer interaction. Also, enhancement in Tat–NFκB binding was observed in the presence of $ZnCl_2$ (Fig. 2D).

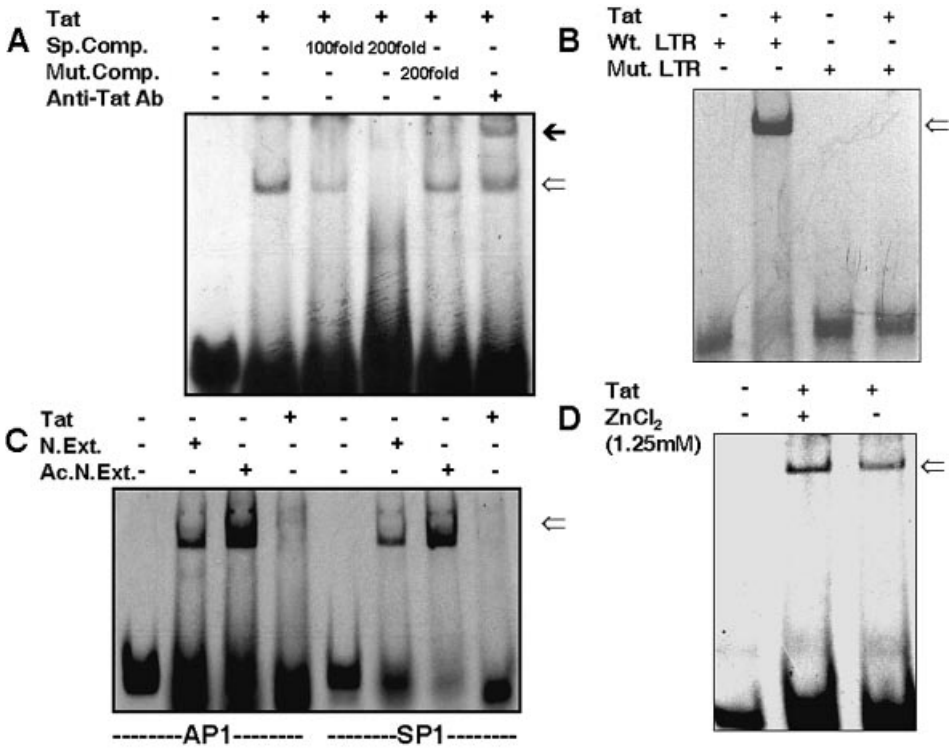

Figure 2. Tat interacts specifically with NFκB enhancer sequences. (**A**) Gel shift assay using NFκB consensus oligonucleotide as labeled probe and cold specific and mutated oligonucleotide as competitors. The sequences are given in Table 1. Lane 1, free probe; lane 2, Tat protein; lane 3, 100-fold excess of cold specific oligonucleotide; lane 4, 200-fold excess of cold specific oligonucleotide; lane 5, 200-fold excess mutated oligonucleotide; lane 6, supershift with anti-Tat antibody. (**B**) Gel shift assay using wild-type and mutated HIV-1 LTR NFκB oligonucleotides (Table 1) as labeled probe. Lane 1, free wild-type oligo; lane 2, Tat; lane 3, free mutated oligo; lane 4, Tat. (**C**) Gel shift assay using AP1 and SP1 consensus oligonucleotides (Table 1) with Tat protein and Jurkat nuclear extracts. Lanes 1–4 depict use of AP1 and lanes 5–8 shows use of SP1 consensus oligonucleotide. Lane 1, free probe AP1; lane 2, nuclear extract of Jurkat; lane 3, nuclear extract from activated Jurkat; lane 4, Tat; lane 5, free probe SP1, lane 6, nuclear extract of Jurkat; lane 7, nuclear extract from activated Jurkat; lane 8, Tat. (**D**) Gel shift showing enhancement of Tat binding to HIV-1 LTR NFκB oligonucleotide in the presence of 1.25 mM $ZnCl_2$. Lane 1, free probe; lane 2, Tat (500 ng) in presence of Zn^{2+} cations; lane 3, Tat (500 ng).

In order to check whether NFκB enhancer sequence could bind to Tat in the presence of NFκB protein in nuclear extract, we then studied the binding of LTR-NFκB probe with the nuclear extract prepared from Jurkat-Tat cells expressing Tat endogenously. The Jurkat-Tat nuclear extract gave shifted complexes with LTR-NFκB probe, which seems to comprise both Tat and NFκB bound shifted complexes, as evidenced by supershift with both Tat and p65 (NFκB) antibody (Fig. 3A). In the control experiment only an NFκB-mediated shift was observed in untransfected Jurkat nuclear extract (Fig. 3B), whereas Tat antibody did not show any supershift (Fig. 3C). Finally, only wild-type Tat (1–86 amino acids) protein could bind to the LTR NFκB probe while a transactivation negative mutant of Tat C22G failed to interact, indicating again the specificity of the binding of Tat protein to the NFκB enhancer sequence (Fig. 3D).

In order to verify this NFκB enhancer DNA and Tat protein interaction independently, we also screened an oligonucleotide library using SELEX technology. Iterative screening of the oligonucleotide library was carried out to determine the consensus binding motif for Tat protein. The sequences internal to the primer in the selected sequences (Table 3) were analyzed using the motif discovery tool MEME (Multiple EM for Motif Elicitation) software version 3.0 (http://meme.sdsc.edu/) (38) to identify a 15 bp motif bound by Tat protein with the experimentally obtained sequences. The multilevel consensus sequence generated by the matrix obtained from the software is shown in Figure 4, which corresponds closely to the complementary NFκB consensus enhancer sequence. The above interaction was further confirmed by recording CD spectra of Tat protein alone and after incubation with LTR NFκB wild-type and mutated oligonucleotides. We observed a negative band at 208 nm, which on addition of wild-type LTR oligonucleotide was reduced in intensity (Fig. 5). This change is not observed with mutated oligonucleotide and is also not due to a simple additive effect of the spectra of DNA and protein, since the computer addition spectra were different from the spectra of the complex. This definite change in signature supported specific interaction of NFκB enhancer sequence and Tat protein.

The above mentioned experiments clearly indicate the presence of NFκB enhancer binding activity of Tat protein *in vitro*. Previous transactivation studies using wild-type and TAR-deleted LTR reporter constructs by several laboratories have unequivocally pointed toward the importance of NFκB enhancer sequence of LTR in TAR-independent transactivation (16–19), but the effect of Tat on isolated NFκB enhancer has not been studied. The *in vivo* role of the observed Tat-NFκB DNA binding was established by transient transfection studies in the HEK 293T cell line. A dose-dependent increase

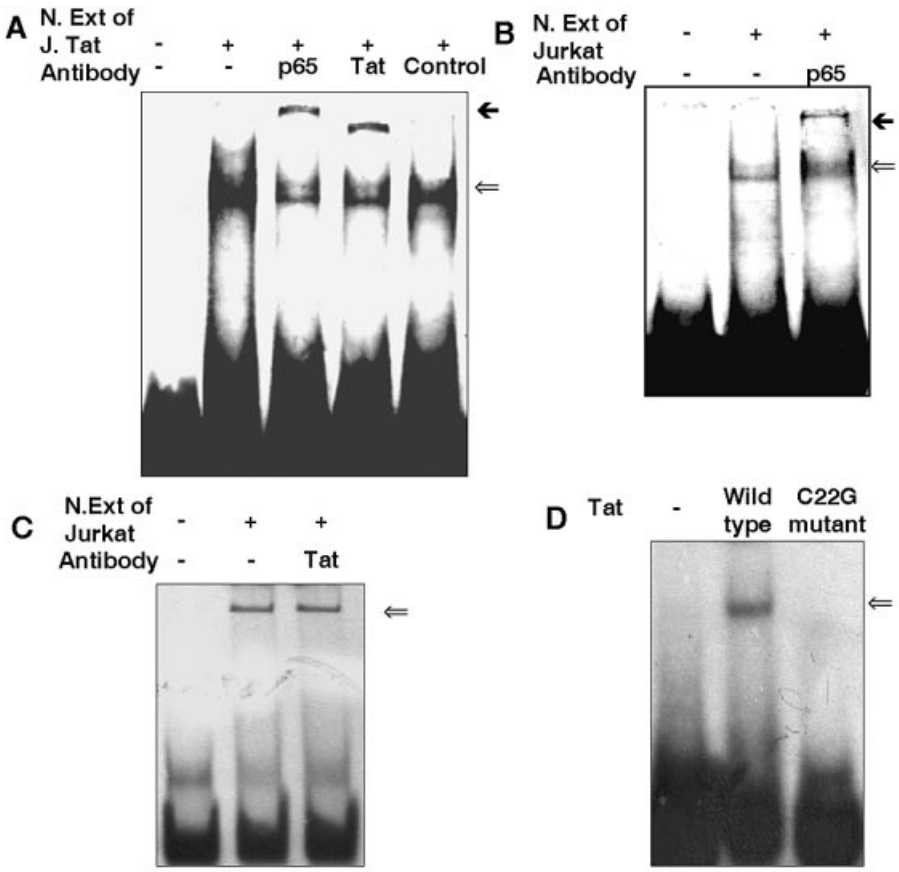

Figure 3. LTR-NFκB enhancer interacts with purified Tat protein and endogenously expressed Tat but not with C22G mutant Tat protein. (**A**) Gel shift assay showing binding of endogenously expressed Tat in Jurkat-Tat nuclear extract to LTR NFκB oligonucleotide. Lane 1, free probe; lane 2, nuclear extract of Jurkat-Tat; lane 3, supershift with anti-p65 (NFκB) antibody; lane 4, supershift with anti-Tat antibody; lane 5, supershift with isotype control antibody. Arrows indicate shifted complexes. (**B**) Gel shift assay showing binding of NFκB complex in Jurkat nuclear extract to LTR-NFκB oligonucleotide. Lane 1, free probe; lane 2, nuclear extract of Jurkat; lane 3, supershift with anti-p65 (NFκB) antibody. Arrows indicate shifted complexes. (**C**) Gel shift assay using LTR-NFκB oligo and Jurkat nuclear extract showing specificity of Tat antibody. Lane 1, free probe; lane 2, nuclear extract of Jurkat; lane 3, nuclear extract of Jurkat with anti-Tat antibody. Arrows indicate shifted complexes. (**D**) Gel shift assay using LTR-NFκB oligonucleotide and wild-type and mutant Tat protein. Lane 1, free probe; lane 2, wild-type Tat; lane 3, mutant Tat (C22G). Arrows indicate shifted complexes.

Table 3. List of oligonucleotide sequences obtained in SELEX experiment using Tat protein as described in Materials and Methods

Sr. no.	Sequence
1	GTGTGTTTCGCAAACAGACGCTGATCCTTAAC
2	CGAATCACACCAACCTGACGCGAAGGGATCGC
3	GTGTGTTTCGCAAACAGACGCTGATCCTTAAC
4	CGAAACACACCAACCTGACGCGAAAGGATCGC
5	GTGTGTTTCGCAAACAGACGCTGATCCTTAAC
6	CGAAACACACCAACCTGACGCGAAAGGATCGC
7	GTGTGGTTCGCAAACAGACGCTGATCCTTAAC
8	CGAACCACACCAACCTGACGCGAAAGGGTCGC
9	GCCGAAGTCTATTAAAGAGACGCTGGAAGGTG
10	AGACGCTGATCCTTAACTTACCCGGTCAGCCG
11	ACCGTGTACCACTAACGTTACCCGCAGCGTCC
12	ACCGTGTACCACTAACGTTACCCGCAGCGTCC
13	GGTGTGATTCGCAAGCAGACGCTGATCCTTAA
14	TACCGTGTACCACAACGTTACCCGCAGCGTCC

of up to ~8-fold in reporter activity (Fig. 6) of NFκB enhancer driven luciferase (pNFκB-luc) construct was observed on co-transfection with Tat expression vector pCDNA-Tat. This activity was obliterated when the reporter plasmid was co-transfected with pCDNA encoding truncated 1–48 Tat mutant (Fig. 6), which is known to be devoid of the nuclear localization signal and nucleic acid binding motif (2). In addition to the TAR-independent transcriptional activation of HIV-1 LTR, the observed increase in the NFκB driven reporter activity is of particular importance as a possible mechanism for Tat-mediated modulation of cellular gene function. In order to identify the presence of Tat binding to NFκB enhancer *in vivo*, we then performed a CHIP assay using HIV-1 NL4.3-infected CEM-GFP cells. The presence of LTR-containing genomic sequences bound to Tat protein was analyzed by formaldehyde cross-linking of HIV-infected cells followed by chromatin immunoprecipitation of HIV-infected cells by Tat antibody. Figure 7A represents the cross-linked DNA–protein complex sheared by sonication (input sample) prior to immunoprecipitation. PCR amplification with LTR specific primers flanking the NFκB enhancer region between nuc-0 and nuc-1 yielded a specific 175 bp product in chromatin immunoprecipitated with Tat antibody, while no detectable product band was found with isotype antibody control (Fig. 7B). To rule out any nonspecific amplification, a

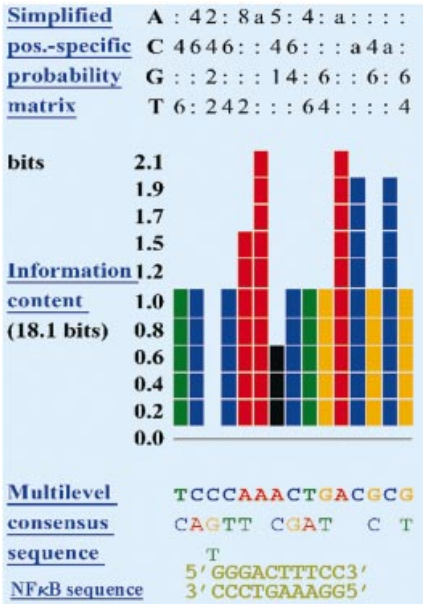

Figure 4. Position-specific matrix generated by MEME software analysis. DNA sequences obtained by oligonucleotide library screening were analyzed as described in the text. The matrix shows the bit score for the occurrence of a particular nucleotide in the motif, total bits score as calculated and consensus obtained. The sequence derived is complementary to NFκB enhancer-like sequence.

control PCR was carried out using HIV-1 nuc-1 and nuc-2 region specific primers, which show a specific PCR product in input control while no band was detected in anti-Tat and isotype control (Fig. 7C). This experiment conclusively shows the binding of Tat protein to the enhancer region of the LTR integrated in chromatin and supports an important role for Tat in chromatin remodeling as reported earlier (31).

DISCUSSION

Multiple regulatory elements are required for activation of HIV-1 LTR. The activation is dominated by the Tat–TAR interaction; however, TAR-independent activation has also been widely reported for Tat, indicating an alternative mechanism for Tat functions (16–19). In addition to the well characterized ability of Tat to interact with a variety of cellular proteins, some previous reports indicate that Tat might also act on cognate DNA sequences in HIV-1 LTR and thereby regulate viral gene expression (18,22,23). Using Gal4 DNA binding domain fusion of Rel A and Tat protein, Yang *et al.* (39) have suggested an alternative regulatory pathway for Tat transactivation in specific cells derived from the central nervous system. They and others have been able to show convincingly the importance of NFκB and SP1 enhancer elements in the LTR for TAR-independent transactivation by Tat (18). Their data indicate that tethering of Tat onto the enhancer region of LTR-containing NFκB and SP1 elements is critical for TAR-independent transactivation (39). Also, Tat is unable either to activate transcription or to induce changes in the chromatin structure of integrated proviral promoter lacking both SP1 and NFκB sites (25). However, no direct

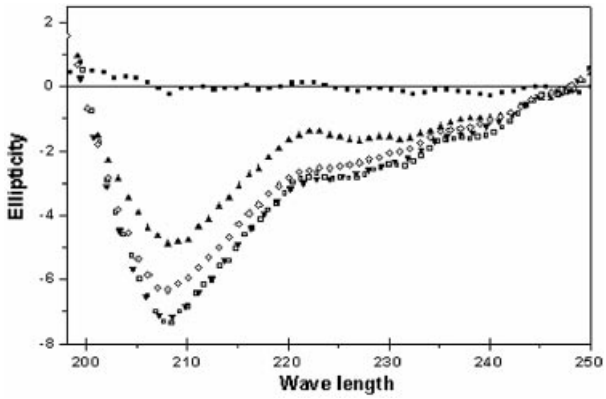

Figure 5. CD spectra of Tat protein in the presence of LTR-NFκB oligonucleotide: solid triangles (down), CD spectrum of Tat protein, 30 μg/ml in PBS pH 7.4; solid triangles (up), change in CD signature of Tat protein in the presence of LTR-NFκB oligonucleotide, 0.1 nmol; open diamonds, change in CD signature of Tat protein in the presence of mutant LTR-NFκB oligonucleotide, 0.1 nmol; solid squares, CD signature of LTR-NFκB oligonucleotide, 0.1 nmol; open squares, computer addition spectra of LTR-NFκB oligonucleotide and Tat protein.

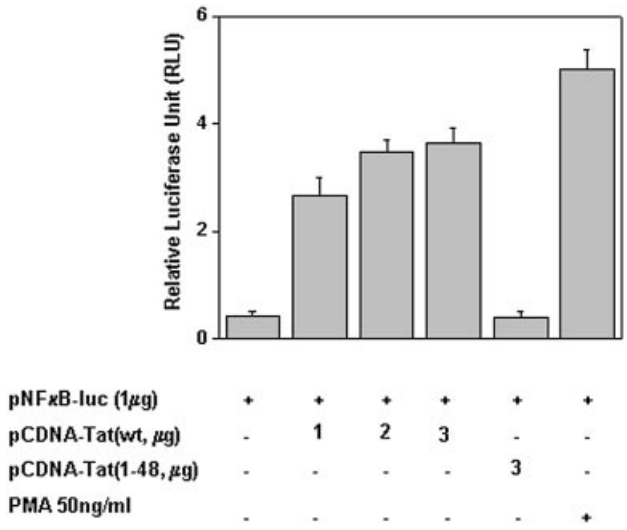

Figure 6. HIV-1 Tat protein activates NFκB enhancer driven reporter gene expression. HEK 293T cells were transfected with pNFκB-luc (1 μg) reporter vector (Stratagene, USA) together with either pCDNA-Tat or pCDNA-Tat (1–48), or were stimulated with 50 ng/ml PMA. pEGFPN1 vector (Clontech, USA) was co-transfected in all experiments for normalization based on GFP expression. Luciferase assays were performed 36 h post-transfection using a Luclite™ assay kit on a TopCount microplate counter (Packard Bioscience, USA). The normalized data shown represent the mean + SEM of three independent experiments.

DNA binding activity has been shown with native Tat protein and the proposed cognate target remains to be identified.

An initial structural homology search of the Tat protein sequence using the hidden Markov model (37) indicates that HIV-1 Tat shows maximal homology with mouse and human p50 protein, a member of the NFκB family of transcription factors. Then our gel shift data clearly indicate that, like other DNA binding transcription factors, Tat binds to NFκB enhancer elements of the LTR promoter sequences and

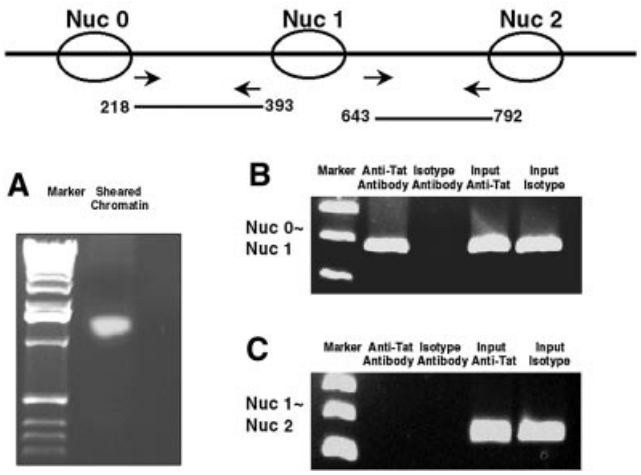

Figure 7. CHIP assay showing interaction of Tat with the enhancer region of the integrated LTR *in vivo* in HIV-1-infected CEM-GFP cells. HIV-1 NL4.3-infected CEM-GFP cells were cross-linked by 1% formaldehyde followed by quenching with 125 mM glycine. Cells were washed and the pellet was lysed, followed by sonication. The sonicated lysate was pre-cleared with protein A/G beads, salmon sperm DNA and BSA. The cleared lysate was immunoprecipitated with anti-Tat antibody or isotype control by incubation at 4°C overnight. The chromatin antibody complex was immobilized on protein A/G beads and then eluted in 2% SDS, 0.1 M $NaHCO_3$ and 10 mM DTT. Cross-links were reversed and the protein was digested with proteinase K (100 μg/ml). DNA was recovered by phenol–chloroform extraction and ethanol precipitation. Precipitated DNA was PCR amplified using LTR specific primers (Table 2) and products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining. (**A**) Chromatin isolated after cross-linking of infected cells was sheared by sonication, run on agarose gel and visualized by ethidium bromide staining (M, marker) Lane 1, sheared chromatin. (**B**) HIV-1 LTR enhancer region between Nuc-0 and Nuc-1 specific PCR product (region: 218–393 of NL4.3) obtained by CHIP using antibody against Tat protein. Lane 1, Tat IP; lane 2, isotype control; lane 3, Tat input control; lane 4, isotype input control. (**C**) HIV-1 LTR enhancer region between Nuc-1 and Nuc-2 specific PCR product (region: 643–792 of NL4.3) obtained by CHIP using antibody against Tat protein. Lane 1, Tat IP; lane 2, isotype control; lane 3, Tat input control; lane 4, isotype input control.

could also bind specifically to the canonical NFκB enhancer sequence, pointing thereby to its possible role in modulating cellular gene promoters. Tat has already been shown to modulate the expression of several cellular genes (6,21) in the absence of TAR-like RNA sequences and thus the NFκB binding shown here could be one of the mechanisms involved in the modulation. Specificity of this interaction is obvious as not only does Tat not bind to canonical SP1 and AP1 sequences which are important elements of LTR-mediated gene expression, but also Tat C22G mutant failed to bind the NFκB enhancer sequence in the LTR. CD spectra of Tat showed the random coil type secondary structure of protein. Although no significant rearrangement of secondary structure was noticed on binding to DNA, a definite and reproducible change in CD signature was observed. This also confirmed the definite interaction between Tat and DNA. Furthermore, Tat-induced activation of NFκB enhancer driven luciferase expression provides functional relevance to this interaction. Finally, an *in vivo* interaction of Tat with integrated LTR in the chromatin definitely points toward a regulatory role for this binding in viral gene expression. The role of Tat in both initiation and elongation of transcription has been clearly deciphered (2,3,10). Through its multifaceted activity, Tat has been shown to be important in histone modification which is essential for gene expression and reactivation from latency. Tat is shown to interact with HATs like p300 and P/CAF (11–15), which have pleiotropic functions in chromatin modulation and gene regulation. Interestingly, it has been proposed in a recent report (40) that the Tat protein in the monocytic U1 cell line is able to recruit P/CAF to promoter but lacks its own transactivation function, resulting in a basal level of gene expression. Addition of Tat protein *in trans* resulted in enhanced reactivation of virus from latency. In this context, a direct interaction of Tat with LTR could be hypothesized for enhanced recruitment of HATs to viral promoter resulting in increased transcription initiation.

Earlier reports have clearly demonstrated that Tat interacts with SP1 (41–43) and also with cyclin T1 (9,44) and thus may synergize to enhance the level of transactivation. Recently the role of SP1 in recruiting cyclin T1 has been elucidated (45). Thus, based on these previous reports, it could be said that Tat might help in stabilizing the transcriptional complex by bringing cyclin T1 in close proximity to SP1 or tethering of cyclin T1 to the LTR promoter. This also indicates that SP1 plays an important role in LTR-mediated gene expression and synergizes with Tat and may tether it to the enhancer region of the promoter (41). Our results showing Tat–DNA interaction could be important not only in the case of cells of neuronal origin where TAR-independent transactivation by Tat has been shown to have a profound effect (16,17,39), but also in transcription initiation from the integrated provirus in other cells. TAR-independent transactivation may also be important due to various cell type specific factors, which may aid in tethering Tat to chromatin. In light of this new information, we propose that Tat modulates TAR-independent transactivation by binding to NFκB enhancer sequences, and it is possible that SP1, by binding to Tat protein, could help in tethering Tat to its binding site on the LTR. Thus Tat binding specifically to chromatin enhancer sequence elements could be the basis not only for TAR-independent transactivation of HIV-1 LTR but also for modulation of cellular gene promoters. Finally, this interaction adds a new paradigm to an increasing list of pleiotropic activities of the Tat protein.

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