Studies on DNA-Protein interactions: HIV-1 Tat as a novel DNA binding transcriptional activator

by

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CERTIFICATE

This is to certify that the work presented in this thesis entitled "**Studies on DNA-Protein interactions: HIV-1 Tat as a novel DNA binding transcriptional activator**" submitted by **Dineshkumar Haribhau Dandekar** was carried out by the candidate at National Chemical Laboratory, Pune, under our supervision. Such materials as obtained from other sources have been duly acknowledged in this thesis.

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CANDIDATE'S DECLARATION

I hereby declare that the thesis entitled "**Studies on DNA-Protein interactions: HIV-1 Tat as a novel DNA binding transcriptional activator**" submitted for the degree of Doctor of Philosophy in Biotechnology to the University of Pune has not been submitted by me to any other university or institution. This work was carried out was carried at National Chemical Laboratory, Pune, India.

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To my Parents, Sisters and Teachers

Abstract

HIV-1 is the causative agent of AIDS. The expression of the viral genome, which encodes for various regulatory, structural, and accessory proteins, is modulated by regulation of viral LTR promoter. Tat protein of HIV-1 is the most important regulator of LTR mediated gene expression. Tat reprograms 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). In addition, several studies have shown convincing evidences that Tat can transactivate HIV-1 LTR mediated gene expression in absence of TAR. It has also been shown that regulatory elements in the enhancer region of LTR play an important role in TAR independent transactivation gene of expression but the molecular basis of this activation remains to be clearly understood. Although the previous reports point towards the possibility of a DNA binding activity for Tat, it has not been verified experimentally and is the basis of the present work.

This thesis demonstrates a direct and specific interaction of Tat with nuclear factor kappa B (NFκB) enhancer DNA sequence, which has been shown to be important for both TAR independent and dependent Tat responsive transactivation of HIV-1 LTR, *in vitro* and *in vivo*. This interaction leads to transactivation of gene under control of NFκB enhancer and provides a novel molecular basis to explain TAR-independent transactivation in HIV-1.

Furthermore, the Tat-DNA interaction was characterized using various biophysical methods. Quantitative fluorescence anisotropy was utilized to determine stoichiometry and equilibrium binding constants for Tat: NFκB enhancer DNA interaction. Anisotropy studies using fluorescein labeled DNA suggests that Tat binds to NFκB enhancer DNA as a dimer

with binding affinity (K_d; dissociation constant) in nanomolar range. This binding is specific at physiological salt concentration and is associated with large heat changes that were measured by isothermal titration calorimetry (ITC).

Tat is multifunctional protein which plays a major role in HIV-1 pathogenesis. Several studies have shown convincing evidence that Tat can induce remodeling of proviral LTR chromatin by recruiting histone acetyl transferases, which results in histone acetylation. Tat is known to modulate many cellular genes and make cellular environment more favorable for the survival of the virus. NFκB enhancer element, the identified DNA target for Tat protein, is a regulatory sequence in promoter of many cellular genes. Hence it was envisaged that Tat-DNA interaction could be the potential mechanism of Tat- mediated modulation of cellular genes in infected. Studies on this line show that Tat is recruited to Interleukin-8 and Interleukin-2 gene promoter in infected cells as confirmed by chromatin immunoprecipitation assays. A temporal correlation of Tat recruitment with HDAC recruitment and acetylation of histones was observed at both LTR as well as Interleukin-2 gene promoter during course of viral infection. This appears to be a significant phenomenon which was hitherto not explored and adds an additional level of control in regulation of viral expression. There appears a dynamic interplay between host factors and viral Tat protein which leads to variation in gene expression.

Abbreviations

ΔG	Change in Gibbs energy
ΔH	Change in enthalpy
ΔS	Change in entropy
μg	Microgram
μM	Micromolar
AcH-3	Acetylated Histone-3
AcH-4	Acetylated Histone-4
AIDS	Acquired immunodeficiency syndrome
AP1	Activator protein 1
APOBEC	apolipoprotein B mRNA editing enzyme, catalytic polypeptide
ARV	AIDS associated retrovirus
BSA	Bovine serum albumin
C	Celsius
CA	Capsid
CBP	CREB binding protein
CCR5	Chemokine (C-C motif) receptor 5
Cd	cadmium
CD	Circular dichroism
CD4	Cluster of differentiation 4
CDK9	Cyclin dependent kinase 9
cDNA	complementary DNA
ChIP	Chromatin immunoprecipitation
CNS	Central nervous system
CRF	Circulating recombinant forms
CRM1	Chromosome region maintenance protein 1
C-terminal	Carboxy terminal
CXCR4	Chemokine (C-X-C motif) receptor X4
CyPA	Cyclophilin A

DBD	DNA binding domain
DMEM	Dulbecco's modified Eagle's medium
DRB	5,6-Dichlorobenzimidazole 1-β-D-ribofuranoside
DTT	Dithiothreitol
EDTA	Ethylenedinitrilotetraacetic acid
ELISA	Enzyme linked immunosorbent assay
env	Envelope gene
FCS	Fetal calf serum
gag	Group specific antigen gene
GFP	Green fluorescent protein
gp120	Glycoprotein 120
gp41	Glycoprotein 41
GST	Glutathione S transferase
H-3	Histone-3
H-4	Histone-4
HAT	Histone acetyltransferase
HIV	Human immunodeficiency virus
HIV-1	HIV subtype 1
HIV-2	HIV subtype 2
HTLV	Human T cell leukemia virus
IL-2	Interleukin 2
IL-8	Interleukin 8
IN	Integrase
ITC	Isothermal titration calorimetry
K	Kelvin
Kb	Kilobases
Kcal	Kilocalories
K_d	Dissociation constant
LAV	Lymphadenopathy virus
LBP-1	Leader binding protein-1
LSF	Late SV40 Factor

LTR	Long Terminal Repeat
M	Molar
m	Meter
MA	Matrix
MHC	Major histocompatibility complex
MHR	Major homology region
min	Minutes
mL	Milliliter
mM	Millimolar
MnSOD	Manganese super oxide dismutase
MOI	Multiplicity of infection
mRNA	messenger RNA
NC	Nucleocapsid
Nef	Negative factor
NFκB	Nuclear factor kappa of B cells
NFAT	Nuclear factor of activated T cells
NLS	Nuclear localization signal
NMR	Nuclear magnetic resonance
NP40	Nonidet® P40
nt	Nucleotide
N-terminal	Amino terminal
nuc	Nucleosome
P/CAF	p300/CREB associated factor
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PIC	Preintegration complex
PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenylmethylsulphonyl fluoride
pol	Polymerase gene
PR	Protease
pTEFb	Positive transcription elongation factor b

qChIP	Quantitative chromatin immunoprecipitation
RNA pol II	RNA polymerase II
ROS	Reactive oxygen species
RPMI	Roswell park memorial institute
RRE	Rev responsive region
RT	Reverse transcriptase
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SELEX	Systematic evolution of ligands by exponential enrichment
SP1	Stimulatory protein 1
SU	Surface protein (gp120)
TAK	Tat activated kinase
TAR	Transactivation response region
Tat	Transactivator
TM	Transmembrane (gp41)
TRAIL	TNF related apoptosis inducing ligand
V	volts
Vif	Viral infectivity factor
Vpr	Viral protein R
Vpu	Viral protein U
YY1	Ying yang 1
Zn	Zinc

Chapter 1: Introduction

1.1 Discovery of HIV-1

In early 1980s a rare kind of disease was observed in active homosexuals and intravenous drug users. This was later named as Acquired Immuno Deficiency Syndrome (AIDS). Clinical and epidemiological investigation provided persuasive evidence that disease was caused by an infectious agent. The cause was probably an unidentified virus. Since its discovery, AIDS has been intensely investigated by many scientists. Although the cases of AIDS were identified only in 1980s, it was found to be present in patients samples dating back to early 1950s.

Early search on causative agent of AIDS lead to tremendous increase in research activity and isolation of etiological agent by three groups. Luc Montagnier and Robert Gallo identified this agent as lymphadenopathy -associated virus; LAV (1) and Human T cell leukemia virus III; HTLV III (2), followed by report of characterization AIDS associated retrovirus; ARV by Jay Levy (3). These groups later recognized that the identified virus shared a common genome and belongs to genus *Lentivirus*, (Latin *lentus*, meaning slow) family *Retroviridae*, having distant homology to Human T cell leukemia virus. Although there was obvious homology, these viruses were shown to have some distinct properties not found in HTLV and hence were renamed Human immunodeficiency virus; HIV (4).

1.2 Epidemiology of AIDS

The origin of HIV, which has a relatively complex genome, remains an issue of considerable debate (5).The progenitor virus is commonly believed to be transferred from primate in near past, but few others have suggested its evolution from virus which existed

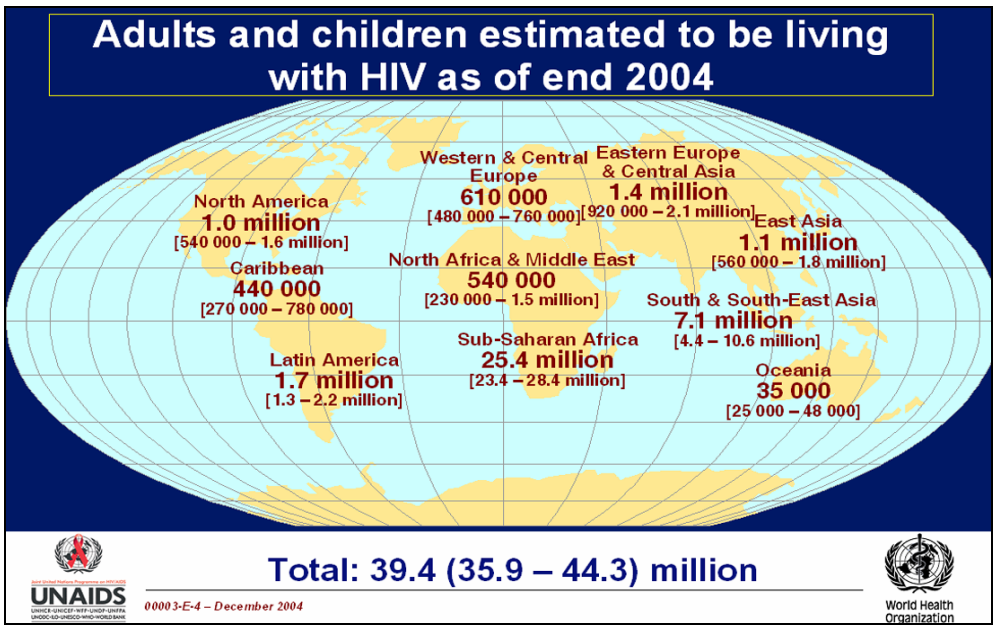


Figure 1: Status of the AIDS pandemic. Estimate for number of people infected worldwide by the end of the year 2004. The ranges in parenthesis define the boundaries for actual numbers. (Source: UNAIDS)

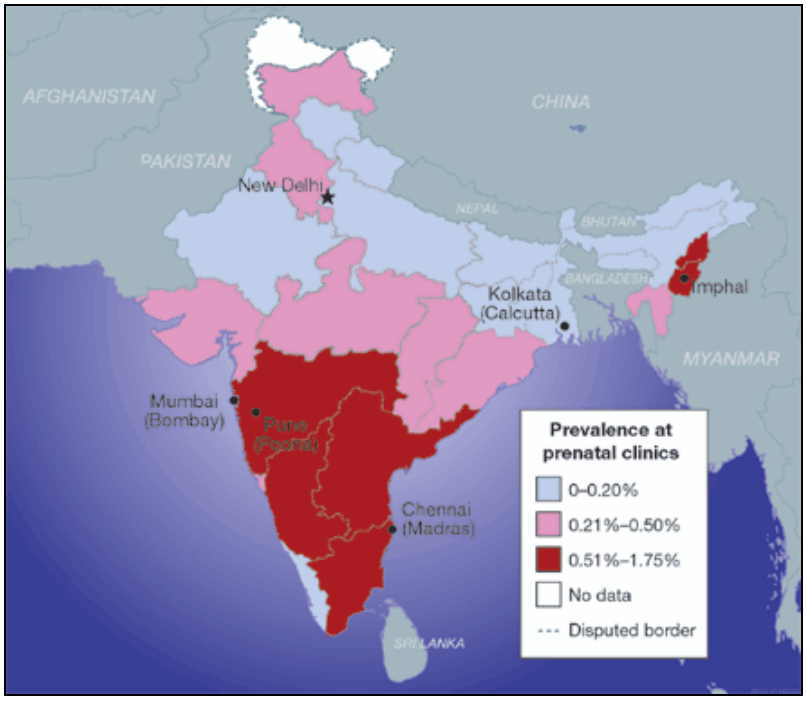


Figure 2: Current status of HIV-1 prevalence in India (Source: Cohen, J. (2004) *Science,* **304**: 504- 509.)

several thousand years ago (6). The disease is characterized by severe immunodeficiency due to profound decrease in the number of $CD4^{+ve}$ T cells and susceptibility to infection with opportunistic pathogens. The majority of people infected with HIV, if not treated, develop signs of AIDS. Soon after the discovery of primary agent of AIDS, HIV-1, a second type of virus also causing immunodeficiency was discovered and named as HIV-2. Both HIV-1 and HIV-2 have the same modes of transmission and are associated with similar opportunistic infections. In persons infected with HIV-2, development of immunodeficiency and progression to AIDS is slower when compared with persons infected with HIV-1.

The spread of HIV and its impact has been far greater than anticipated. In particular it poses a major challenge to developing world. There has been much success in improving the quality of life of AIDS patients due to recent therapies. But much desired is yet to be achieved. AIDS constitutes one of the major health challenges to medical community not only India but across the globe (Figure-1 and 2). Responding to HIV/AIDS epidemic is now a global imperative.

1.3 Mode of transmission

Infection with HIV generally occurs after transfer of body fluids from an infected person to an uninfected one. The virus is carried in infected $CD4^{+ve}$ T cells, dendritic cells, and macrophages, and as a free virus in blood, semen, vaginal fluid, or milk. HIV most commonly spreads by sexual intercourse, contaminated needles used for intravenous drug delivery, and the therapeutic use of infected blood or blood products. Mothers transmit HIV *in utero*, or at the time of birth, or by breastfeeding milk to their infants. Transmission by

blood products has largely been eliminated since introduction of screening at blood banks for the presence of HIV.

1.4 Human Immunodeficiency Virus –1

HIV-1 is an enveloped retrovirus with a complex life cycle. HIV-1 virion exhibits cone-shaped capsid core particles enveloped by a lipid bilayer that is derived from the membrane of the host cell. The HIV-1 genome is encoded by a ~ 9.8 Kb RNA, which is packaged in the virion particle. The genome encodes nine open reading frames (Figure-3) (7). Three of these encode the Gag, Pol, and Env polyproteins, which are subsequently proteolyzed into individual proteins. The four Gag proteins, matrix (MA), capsid (CA, p24), nucleocapsid (NC, p7), and p6, and the two Env proteins, gp120 (surface or SU) and gp41 (transmembrane or TM), are structural components that make up the core of the virion and

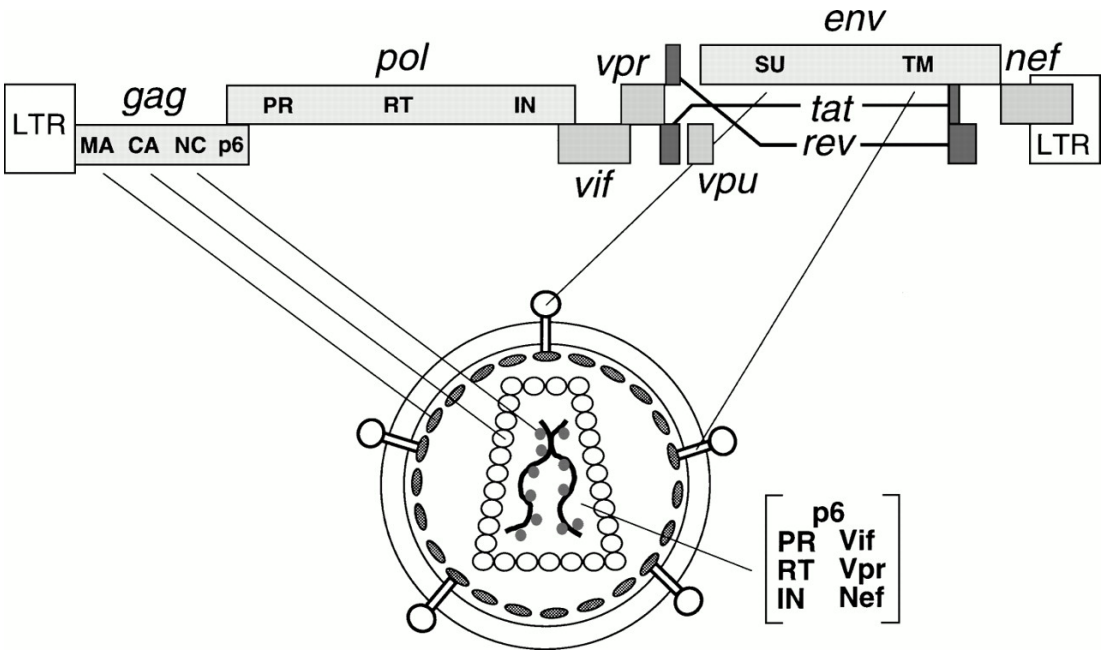


Figure 3: Organization of the HIV-1 genome and virion. (Source: Frankel, A and Young, J. A. T. (1998), *Annual Review Biochem*., **67**: 1-25.)

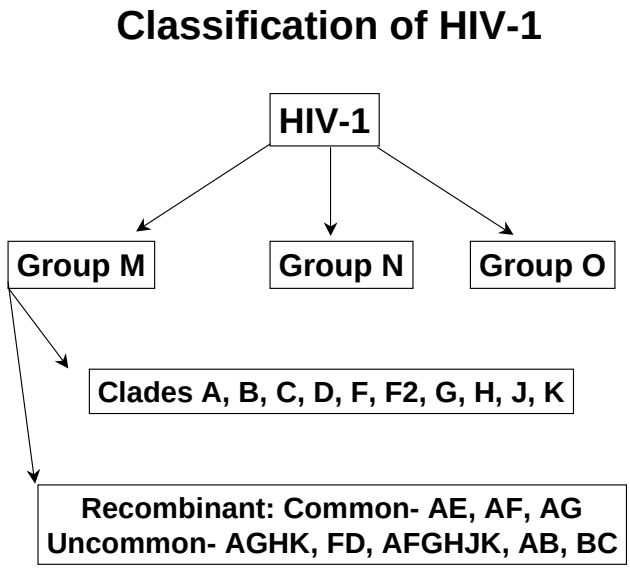


Figure 4: Classification of the different clades of HIV-1

outer membrane envelope. The three Pol proteins, protease (PR), reverse transcriptase (RT), and integrase (IN), provide essential enzymatic functions and are also encapsulated within the particle. HIV-1 encodes six additional proteins, Vif, Vpr, Nef, Tat, Rev, and Vpu. Thus, in simplistic terms, HIV-1 may be considered as a molecular entity consisting of 15 proteins and RNA (7).

1.4.1 Subtypes of HIV-1

HIV-1 can be classified into three groups: the "major" group M, the "outlier" group O and the "new" group N (8). Major prevalence of HIV-1 infections belongs to group M. There are at least nine genetically distinct subtypes (or clades) of HIV-1 at present within group M. These are denoted as subtypes A, B, C, D, F, G, H, J and K (Figure-4). The HIV-1 subtypes are very unevenly distributed throughout the world, with the most widespread being subtypes B and C. Historically, subtype B has been the most common subtype in

Europe, the Americas, Japan and Australia. Subtype C is largely predominant in southern and eastern Africa, India and Nepal. In recent times subtype C has taken over as the most prevalent clade worldwide.

Groups O and N currently represent only a small number of characterized strains. Sequence analysis of *env* and *gag* region of group O isolates has demonstrated that they belong to distinct phylogenetic clusters and have similar diversity like group M subtypes. Occasionally, two viruses of different subtypes can be present in same infected person and recombination of their genetic material creates a new hybrid virus. Many of these new strains do not survive for long, but those, which infect more than one person, are known as "circulating recombinant forms" or CRFs.

1.4.2 Life Cycle of HIV-1

HIV-1 has extraordinary capacity to exploit the cell's molecular machinery during the course of infection. Detailed analysis of the molecular biology of HIV is the key to understanding the mechanisms by which this virus persists in host and causes AIDS, and also for developing effective antiretroviral strategies. *In vivo* viral life cycle is a dynamic multi-step process, which includes infection of target cells, replication, release of virion from infected cells and subsequent infection of new target cells (Figure-5) (9).

The journey of virus inside the host begins with infection of target cell. The virion gains entry into the cell by a receptor mediated mechanism. The binding of viral coat protein gp120 to CD4 receptor and CXCR4 or CCR5 co-receptor (depending on cell type) leads to conformational change of envelope proteins and the virion fuses with the host cell membrane and delivers its contents. Once the virus has entered the cell several intra cellular

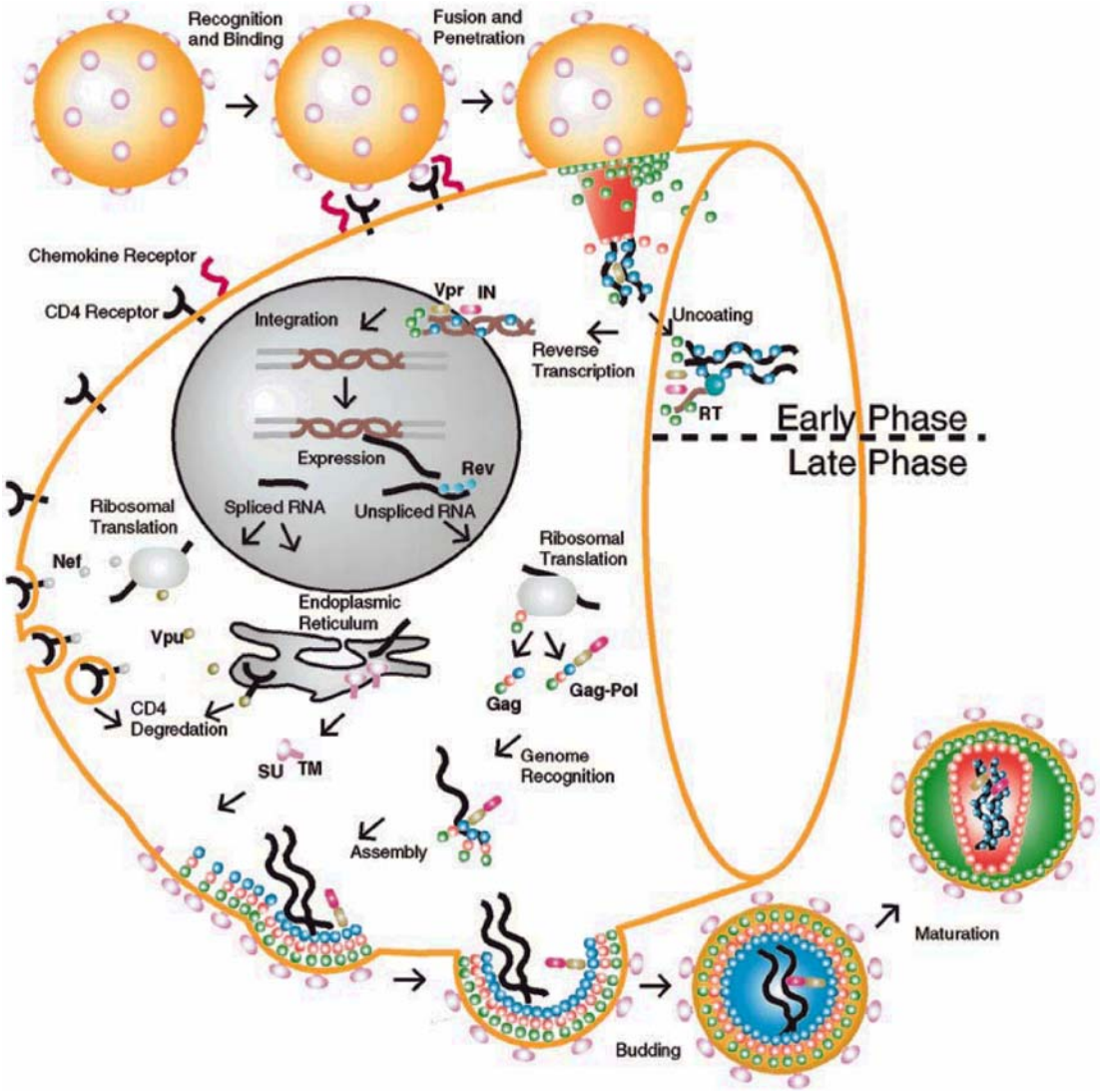


Figure 5: Events in the HIV-1 Life cycle.1) HIV-1 attaches with the help of gp120 molecule, 2) followed by fusion of the receptor CD4 and the chemokine co-receptor, 3) Uncoating of viral capsid takes place in host cells 4) subsequently there occurs reverse transcription and the transfer of genetic information into DNA molecules, 5) the formation of pre-integration complex which involves the complex of DNA with viral and cellular proteins facilitating the entry of the HIV-1 DNA into the nucleus where it integrates into the host chromosome 6) the integrated provirus express the viral mRNA 7) the Rev protein facilitates the export of mRNA molecules to the cytoplasm following which there is transcription of viral proteins, 8) followed by assembly of new virion, 9) these assembled virions bud from cell membrane 10) which is followed by maturation of these virion. (Source: Turner, B. G. and Summers, M. F. (1999) *J. Mol. Biol.,* **285**: 1-32.)

events take place that lead to establishment of provirus. The capsid disintegrates and the HIV RNA bound by proteins is released into the cytoplasm. The viral RNA is copied into DNA by the *pol* gene product reverse transcriptase. The reverse transcription of RNA to DNA is a complex multi-step process. The cDNA forms pre-integration complex (PIC), along with viral and host cell proteins. The PIC then translocates to nucleus where cDNA integrates into host genome.

Integration of HIV viral DNA is a random but very crucial event in retroviral replication and is essential for the production of progeny virus. Integration event offers permanency to HIV in the infected cells, which henceforth functions as a cellular gene regulated by viral long terminal repeat promoter (LTR). The viral mRNA is spliced and transported to cytoplasm where it is translated into viral polyproteins with help of host cellular machinery. A fraction of RNA transcripts are exported as full length genomic RNA, which are subsequently packaged into new virion. The viral proteins are processed into individual protein by the action of protease. The full length RNAs are recognized by viral proteins. This allows the assembly of proteins into viral particles. The export of full length viral RNA and processing of polyproteins are key events in the maturation of viral particles. The mature virus acquires envelope while budding and are later released from the cell surface.

1.5 HIV-1 Long Terminal Repeat Promoter (LTR)

A promoter sequence located upstream of a gene acts as a signal for binding of transcription machinery. (See Box 1 for a brief description of eukaryotic promoter) Promoters are usually regulated by binding of transcription factor to their respective

recognition site present in the regulatory region. Many alternative control elements (transcription factor binding sites) may be present in one promoter, which results in a complex control of gene expression. Transcription factors are proteins which act as signal molecules and lead to assembly of transcription machinery. Transcription factors can be selectively activated or deactivated by other proteins. Transcription factors are classified according to the structure of DNA-binding domain (DBD) they contain (see Box 2).

Due to integration into host genome, LTR functions like a host promoter and provides an excellent model for deciphering regulatory networks involved in the control of mammalian promoters. Viral and cellular transcription factors interact with the LTR, on

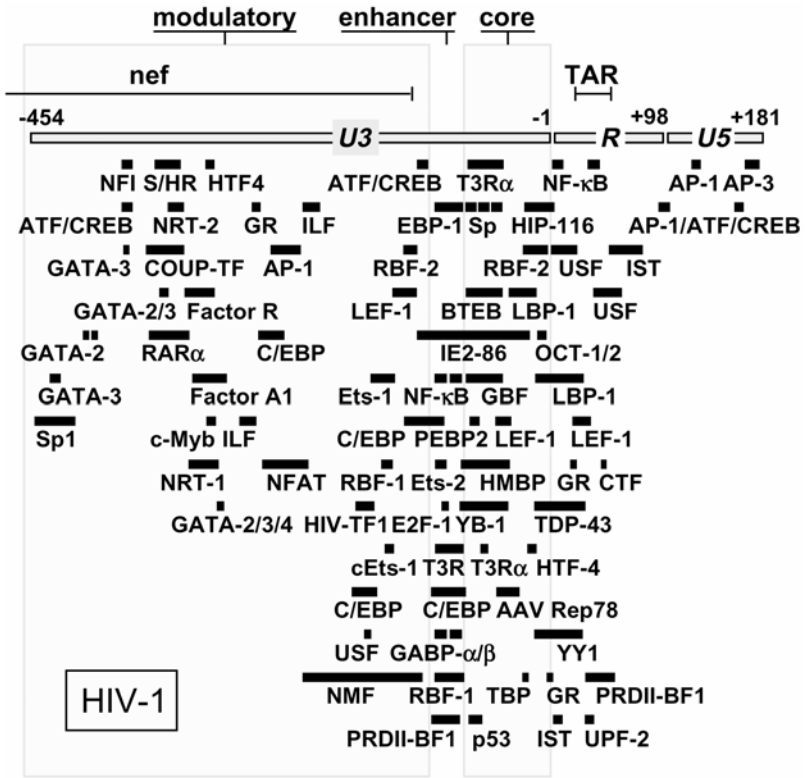


Figure 6: Detailed structure of the HIV-1 LTR. Transcription factors binding sites identified within the HIV-1 LTR by are shown with respect to the structural (U3, R and U5), functional (modulatory, enhancer and core) divisions of the LTR, (Source: Krebs, F. C. *et al*., (2001) in *HIV Sequence Compendium 2001,* pp. 29-70.)

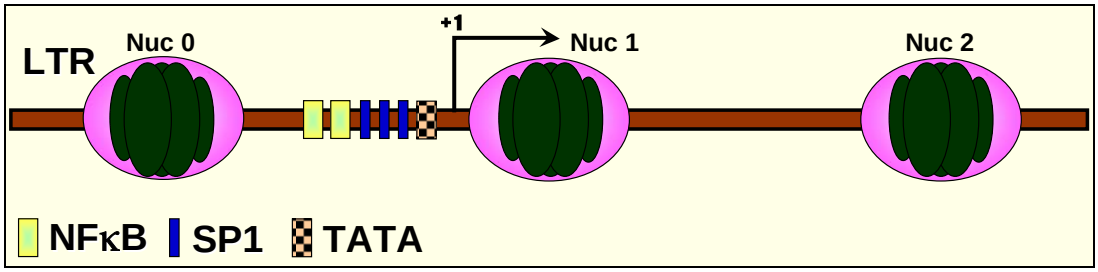


Figure 7: LTR is organized into nucleosomes in vivo. Organization of HIV-1 LTR into positioned nucleosomes (nuc-0 to nuc-2) is depicted. The locations for the binding sites for transcription factors in core promoter and upstream enhancer which are implicated in chromatin-dependent viral gene expression are also shown. Figure not to scale.

their respective binding sites, and are involved in the regulation of HIV-1 LTR. The LTR represents an interesting example of molecular adaptation of a virus to host cellular environment. It can be considered as one of the most complex promoter, which is controlled by many alternative regulatory mechanisms. The viral LTR promoter originates from the unique 5′ and 3′ regions and the flanking R regions of the single stranded RNA genome. The 5′ LTR of provirus serves as the promoter for the entire retroviral genome, while the LTR at the 3′ end encodes the accessory protein, Nef. LTRs contain recognition elements for the basic transcriptional machinery of the host cell.

The overall architecture of the promoter is remarkably conserved among different viral isolates with evidence of some variations in transcription factor binding sites evident in different subtypes (10). The HIV-1 LTR is approximately 640 bp in length and, like other retroviral LTRs, is segmented into the U3 (nt –453 to –1), R (nt +1 to +98), and U5 (+99 to +180) regions. U3 region can be functionally subdivided into the core promoter, the enhancer element, and the negative regulatory element (NRE) (11). In addition to these, sequences downstream of +1 site contain functional binding sites for numerous transcription factors. The plethora of transcription binding sites within the HIV-1 LTR

provides numerous opportunities for cellular and viral transcription factors to interact and regulate LTR activity (Figure-6). Differences in the nuclear milieu specific to cell type and lineage may also modulate LTR activity.

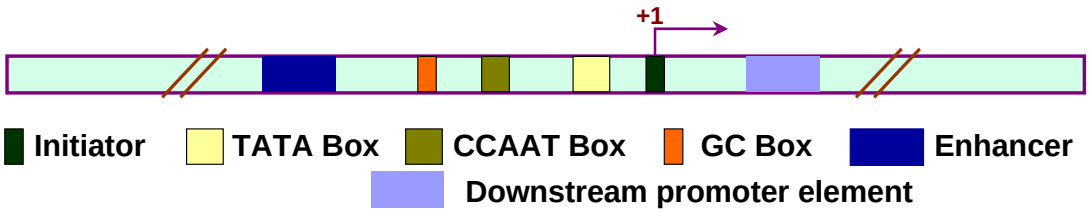
Independent of site of integration into the host genome, the proviral DNA is organized into chromatin. Verdin and co-workers have characterized the precise nucleosome position (Figure-7) in the integrated HIV-1 LTR (12). In a provirus, the LTR is organized into two nucleosome (nuc 0 and nuc 1) which define the two large nucleosome-free regions spanning nt -255 to -3 and +141 to +265. Another nucleosome free region is found downstream of initiation site followed by nuc 2. The nucleosome, nuc-1, which plays a critical role in transcriptional regulation, is located between these two regions. Little information is available about organization of LTR into higher order of chromatin fiber. Sequence analysis and mapping of HIV-1 integration sites in the cellular genome has provided evidence that chromatin structure plays an important role in latency and subsequent activation. The specific roles of viral and host proteins in regulation of integrated LTR have not been fully elucidated. It is well known that HIV-1 regulates the integrated proviral LTR via the pleiotropic effects of its proteins.

LTR promoter is fine-tuned for optimal level of viral replication in infected individual. Transcription is initiated by binding of transcription machinery to the LTR in the core promoter (13). Initiation form LTR is very efficient even in absence of any stimulation. This however results in of synthesis short abortive RNA transcripts. However there is several fold increase in expression of full length viral transcript in presence of Tat.

Box 1...

Transcriptional control from a eukaryotic promoter

Expression from a promoter of eukaryotic gene is generally regulated through multiple *cis*-acting transcription-control regions which can be located either close to the start site (promoter-proximal elements) or may lie at distant places (enhancers). Promoters determine the site of transcription initiation and direct the binding of RNA polymerase II. Genes transcribed by RNA polymerase II have two core promoter elements, the TATA box and the Initiator sequence, that serve as specific binding sites for general transcription factors. The *cis*-acting regulatory sequences are frequently, though not always, located upstream of the TATA box. For example, two regulatory sequences that are found in many eukaryotic genes are located within 100 base pairs upstream of the TATA box: The CCAAT box and the GC box. Some genes may have downstream promoter elements.



General organization of eukaryotic promoter

Promoter-proximal elements occur within ≈200 base pairs of the start site. Several such elements, containing up to ≈20 base pairs, may help regulate a particular gene. They may be also located from 200 base pairs to tens of kilobases upstream or downstream from a promoter, within an intron, or downstream from the final exon of a gene. Enhancers generally range in length from about 50 to 200 base pairs and include binding sites for several transcription factors. The multiple transcription factors that bind to a single enhancer are thought to interact among themselves. The term enhancesome has been coined to describe large nucleoprotein complexes that assemble from transcription factors, as they bind cooperatively to their multiple binding sites in an enhancer. Promoter-proximal elements and enhancers are often cell-type specific, functioning only in specific differentiated cell types.

Genetic and biochemical studies have shown that repressor proteins as well as the more-common activator proteins regulate eukaryotic transcription. While mutation of an activator-binding site leads to decreased expression of the linked reporter gene, mutation of a repressor-binding site leads to increased expression of a reporter gene.

1.6 HIV-1 proteins

1.6.1 Matrix (MA)

MA is a 131 amino acid myristoylated protein (14, 15) also known as p17. MA stabilizes the viral particle by remaining attached to the inner surface of the virion lipid bilayer after viral maturation and also plays an essential role in the incorporation of the Env glycoprotein spikes during virus assembly (16, 17). MA remains associated with the viral PIC (18, 19) and confers nucleophilic properties due to presence of a putative nuclear localization sequence (NLS) (20-23). Additionally, it also has nuclear export signal (NES) that counteracts the NLS during virus production, thus ensuring that Gag is available for virus assembly (24).

1.6.2 Capsid (CA)

CA or p24 is a 231 amino acid protein. It has crucial roles in particle assembly and after entry into a new target cell. In the mature virion, CA forms the shell of the conical core a characteristic of *Lentivirus* (25). The N-terminal domain of CA interacts with the human peptidyl-prolyl *cis-trans* isomerase cyclophilin A (CyPA), which is incorporated into virion (26-28). The C-terminal domain includes a stretch of 20 conserved residues called the major homology region (MHR) (29), which is essential for replication and likely has a crucial role in the retroviral life cycle(30-35). CA is the major viral protein present in the blood during HIV-1 infection and is commonly used as diagnostic marker. Sensitive antigen capture ELISA based kits are commercially available for routine screening of infection in patients.

Box 2

Transcription factors

DNA binding transcription factors contain a variety of structural motifs that interact with specific sequences and often are classified according to DNA-binding domain (DBD) they contain. Most of the DBDs have characteristic consensus amino acid sequence motifs. Several common classes of DNA-binding domains whose three-dimensional structures have been determined are described below.

1) Homeodomain Proteins

Transcription factors with structure homologous to the Drosophila Engrailed protein type of DNA- binding domain are called homeodomain proteins. These proteins share a highly conserved 60-aa region known as the homeobox, e.g., LIM homeobox protein.

2) Zinc-Finger Proteins

A number of different proteins have regions that fold around a central Zn^{2+} ion, producing a compact domain termed as zinc finger. There are several classes of zinc-finger motifs that have been identified. The C_2H_2 zinc finger, most common DNA-binding motifs in eukaryotic transcription factors, binds one Zn^{2+} ion through the two cysteine (C) and two histidine (H) side chains. The C_4 zinc finger, which generally contains only two finger units and bind to DNA as homodimers or heterodimers, has two groups of four critical cysteines that bind a Zn^{2+} ion. The third type of zinc-finger motif, known as the C_6 zinc contains six cysteines which bind two Zn^{2+} ions, folding the region into a compact globular structure, e. g., SP3 transcription factor.

3) Winged-Helix (Forkhead) Proteins

The DNA-binding domains in histone H5 and several transcription factors that function during early development of Drosophila and mammals have the winged-helix motif, also called the forkhead motif, e.g., FOXP1 transcription factor.

4) Leucine-Zipper Proteins/ bZip

Leucine-Zipper proteins bind to DNA as dimers and contain leucines motifs that are required for dimerization. However, many DNA-binding proteins containing other hydrophobic amino acids in these positions are also now identified. The term basic zipper (bZip) now is frequently used to refer to all proteins with these common structural features, e.g., c-Myc transcription factor.

5) Helix-Loop-Helix Proteins

The structural motif is very similar to the basic-zipper motif and contains an N-terminal α-helix with basic residues that interact with DNA, a middle loop region, and a C-terminal region with hydrophobic amino acids. Because of the basic amino acids characteristic of this motif, transcription factors containing this structure are sometimes referred to as basic helix-loop-helix (bHLH) proteins, e.g., MyoD transcription factor.

1.6.3 Nucleocapsid (NC)

The NC is 55 amino acid zinc finger protein. It is required for efficient packaging of viral RNA. Mutations in zinc finger motifs significantly impair HIV-1 particle production (36) and assembly (37, 38). The highly basic NC protein also has non-specific affinity for nucleic acid and assists in various annealing reactions during the viral life cycle (39).

1.6.4 p6

p6, a 52 amino acid protein is a characteristic feature of *Lentivirus*. It is a major phosphoprotein in mature HIV-1 virion. p6 appears to play a role in incorporation of Vpr in the virus (40).

1.6.5 Protease (PR)

The HIV-1 PR, a homodimer with each unit of 99 amino acids, is a novel aspartic protease that functions to cleave the nascent polyproteins during viral replication (41). PR mediated processing of the gag polypeptide occurs within the context of a dense, immature virus core particle and is concomitant with budding. Hydrolysis of viral polyproteins into functional protein products is essential for viral assembly and subsequent activity (42).

1.6.6 Reverse transcriptase (RT)

RT is a hetero-dimer containing a p65 subunit of 560 amino acids, and p50 subunit of 440 amino acids, both derived from the Pol poly-protein (43). RT possesses both RNA-dependent and DNA-dependent DNA polymerization activity and also contains an RNaseH

domain that cleaves the RNA portion of RNA-DNA hybrids. RT is a low fidelity enzyme that catalyzes one of the defining reactions in retrovirus that converts their RNA genomes into double-stranded DNA early post-infection. The high mutation rate of HIV-1 RT (3 x 10^{-5} per cycle of replication) results in HIV-1 populations being highly heterogeneous in sequence. As a consequence, HIV-1 is able to rapidly evade the host immune response and develop resistance to antiviral drugs.

1.6.7 Integrase (IN)

IN is a 288 amino acids enzyme which posses combined 3' exonuclease, endonuclease and ligase activities (44). IN catalyzes a series of reactions to integrate the linear, viral DNA genome into host chromosome. In the first step, IN removes two 3' nucleotides from each strand of the linear viral DNA, leaving overhanging CA_{OH} ends. In the nucleus, IN makes a staggered cleavage in the cellular target DNA. In the second step, the processed 3' ends are covalently joined to the 5' ends of the target DNA. In the third step, which probably involves additional cellular enzymes, unpaired nucleotides at the viral 5' ends are removed and the ends are joined to the target site 3' ends.

1.6.8 gp120 (SU)

The gp120 is a 481 amino acids surface glycoprotein and has trimeric structure (45-48). gp120 is present on the surface of the viral particle, and binds to CD4 and secondary receptors on macrophages and T lymphocytes. This binding leads to fusion of the viral and cellular membranes.

1.6.9 gp41 (TM)

HIV-1 gp41 is a 345 amino acids transmembrane glycoprotein that transverses the lipid bilayer of the virion (49). TM contains an N-terminal fusogenic domain, which mediates the fusion of viral and cellular membranes (50).

1.6.10 Negative factor (Nef)

Nef is a 206 amino acids phosphoprotein (51). Nef protein was originally identified as a dispensable viral negative factor. But its multiple roles in viral life cycle have now become increasingly clear. It appears to perform various important functions like down modulation of CD4 and MHC-I, and increase viral gene expression by interacting with viral and cellular partners (52).

1.6.11 Viral infectivity factor (Vif)

Vif is a 192 amino acids phosphoprotein (53). The function of Vif was unknown until recent papers revealed that it prevents the encapsidation of APOBEC3G (54) and APOBEC3F (55, 56) two potent antiretroviral cytidine deaminases. APOBEC3G and APOBEC3F inhibit the replication of *vif* deleted HIV-1 by deaminating the minus-strand of the viral reverse transcripts, introducing numerous G-A mutations.

1.6.12 Viral protein R (Vpr)

Vpr is a 78 amino acids protein that mediates the nuclear import of PIC into the nucleus particularly in non-dividing cells such as primary macrophages, enhancing viral infection in

these cells (57). It also causes cell cycle arrest and block cells in G2 phase. Since LTR is more active in G2 phase, this facilitates transcription (58).

1.6.13 Viral protein U (Vpu)

Vpu is 81 amino acids integral membrane protein found in large amounts in HIV-1 infected cells, but is not detected in virions (59). Vpu interferes with formation of CD4/Env complexes and destabilizes the CD4 protein in the endoplasmic reticulum (60). Mutation of the *vpu* gene disrupts late steps in virion maturation, with decreased release of HIV-1 and budding into intracellular membranes (61). Vpu also increases susceptibility of HIV-1 infected cells to Fas killing (62).

1.6.14 Rev

Rev is a 116 amino acids protein. It has a nuclear localization sequence (NLS) and a nuclear export sequence (NES) (63). Rev is produced from fully spliced mRNA and functions early in viral infection. It binds to a 240-base region of RNA secondary structure called Rev Responsive Element (RRE) located within the second intron of HIV-1(64). When rev is in the nucleus, it binds to RRE in viral mRNA. The RNA binding domain of Rev is in the same region as NLS. Thus, upon binding to mRNA, the NLS of Rev is masked, exposing only NES. The unspliced mRNA is then exported to cytoplasm through interactions with the NES receptor CRM1 (65). Rev is required for HIV-1 replication as it plays a critical role in viral particle formation.

1.6.15 Transactivator (Tat)

For several years after the discovery of Tat, the transactivator of viral LTR, many hypotheses were put forward to explain its activity. However, the majority of investigators in the HIV field initially believed that Tat interacts either directly, or indirectly, with the promoter to stimulate initiation because most of the known regulatory proteins that control gene expression work at the level of initiation. Tat is one of the few proteins, which regulates gene expression through control of elongation by RNA polymerase II by interacting with RNA element (66, 67). 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 (68).

1.6.15.1 Structure of Tat

Tat is a ≈14 KD, 86-101 amino acids protein of HIV-1, which is the most important regulator of viral gene expression and replication. The Tat sequence has been subdivided into several distinct regions on the basis of its amino acid composition (69): a N-terminal activation region (amino acids 1-19), a cysteine-rich domain (amino acids 20-31), a core region (amino acids 32-47), a basic region (amino acids 48-57), a glutamine-rich region (amino acids 60-72) and second exon (amino acids 73- 102) (Figure-8).

The structure of Tat remains an unresolved puzzle for many structural biologists around the world. Preliminary information about the three-dimensional structure available by nuclear magnetic resonance (NMR) shows that Tat is largely an unfolded protein (70-72). Deletion analysis have identified that first 57 amino acids are necessary and sufficient for

transactivation of LTR by reporter assays (73). The basic region plays a crucial role in nuclear localization necessary for Tat function. The core region has highly conserved motif RKGLGI and is important for Tat acetylation (74) RGD motif plays a crucial role in integrin receptor mediated endocytosis of Tat (75).

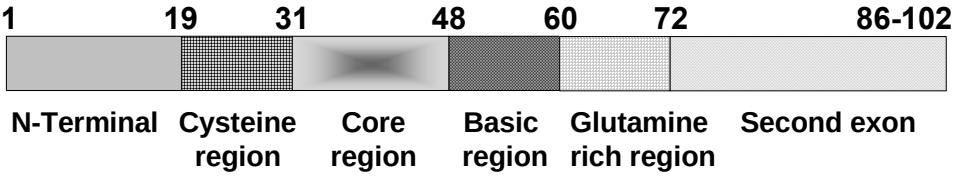


Figure 8: Structural organization of HIV-1 Tat

1.6.15.2 Tat as Transcription factor

Tat has been shown to transactivate 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) (75, 76). In the absence of Tat, initiation from the LTR is efficient, but transcription is impaired because of poor processivity exhibited by the recruited RNA polymerase II that disengages from the DNA template prematurely (77).

After binding to TAR, Tat recruits Tat-activated kinase (TAK) (79, 80)which was later identified as Cyclin dependent kinase 9 (CDK9) and a component of positive transcription elongation factor b (pTEFb) (81) complex, which also contains Cyclin T1(82). The pTEFb complex hyper-phosphorylates RNA polymerase II. This increases the processivity and over 10 –100 fold enhancement in transactivation (83) (Figure-9).

Cyclin T1 plays a critical role in auto-phosphorylation and activation of CDK9; it also increases the specificity of binding and affinity of Tat to TAR RNA (84, 85). The structural

basis for Cyclin T1 interaction with Tat and TAR is still unclear. The most recent models suggest that Cyclin T1 does not bind to TAR directly, but it can alter the conformation of Tat to permit it to recognize the apical loop sequence of TAR RNA in the ternary complex (86). Cyclin T1 is believed to interact with Tat through metal ions stabilized by essential cysteine residues found in both proteins (87, 88). In addition to above, several studies have shown convincing evidence that Tat can transactivate HIV-1 gene expression in the absence of TAR, however the molecular mechanism remains to be clearly elucidated (89-91).

A number of earlier studies clearly indicate that Tat could substantially affect transcription when tethered to DNA (92, 93). Berkhout and co-workers proposed that TAR RNA serves an attachment function directing Tat to the LTR and is only one component of the Tat-responsive target (93). Tat mediated transactivation is efficient only when TAR is present in conjunction with the HIV-1 LTR, NFκB and SP1 DNA sequences as presence of TAR RNA outside this context produced only a suboptimal Tat response. A Tat protein engineered to interact with LTR DNA could transactivate through a TAR-independent

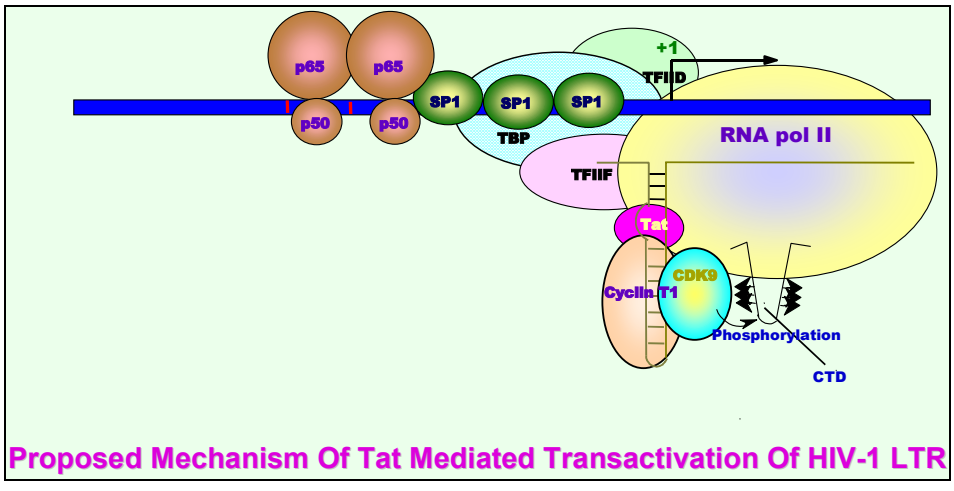


Figure 9: General model for Tat mediated transactivation of LTR. Tat binds to TAR and recruits pTEFb complex that consists of CDK9 and Cyclin T1. This CDK9 then hyper phosphorylates the CTD of RNA pol II. This phosphorylation leads to increased processivity and enhanced expression of viral genes.

mechanism suggesting that Tat also has a DNA target. TAR-independent transactivation by Tat has been shown to have a profound effect in cells of neuronal origin. The transactivational effects of Tat, in CNS-derived cells in general and astrocytic glial cell in particular, are potentially based on the increase of transcriptional initiation, both in TAR-dependent and -independent states. These cells demonstrated profoundly restricted transcription of the HIV-1 genome, which could not be significantly up regulated by various stimulators, such as Phorbol 12-myristate 13-acetate (PMA), tumor necrosis factor-alpha (TNF-alpha) and sodium butyrate. The predominant effect of Tat during TAR-independent transactivation occurs at the level of transcription initiation, whereas a prominent elongation effect of Tat is observed in the presence of TAR. Yang and co-workers suggested an alternative regulatory pathway for Tat transactivation in cells derived from the central nervous system that is independent of TAR and required direct or indirect interaction of Tat with NFκB-binding sites in the HIV-1 LTR (94). There have also been reports establishing functional similarities between Tat and other transcription factors, which enhance the level of gene expression by binding to DNA (95). It is also established that Tat exists as a metal ion (Zn^{2+}or Cd^{2+})-linked dimer bridging cysteine-rich regions of each monomer, a characteristic of DNA binding proteins (96, 97).

Tat is also known to induce chromatin remodeling by recruiting histone acetyl transferases (HATs) such as p300 and p300/CBP associated protein P/CAF to the chromatin, which results in histone acetylation and acetylation of itself (98-102). Tat derepresses the integrated HIV-1 chromatin structure, aiding in activation of transcription of integrated transcriptionally silent HIV-1 promoter. 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, indicating thereby the importance of this region in LTR driven viral gene expression in infected cells (103).

1.6.15.3 Extracellular activities of Tat

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 (104). It has been shown to modulate a number of cellular genes and make the cellular environment amenable for viral replication.

Unique among transcriptional factors of any species, Tat is released in to extracellular milieu (105). Extracellular Tat affects multiple events in the pathogenesis of HIV-1 infection. Tat is secreted from infected cells via a leaderless nonionic Golgi independent secretory pathway (106). This secretion is observed when Tat expression is high and in the absence or low level of cell death (107). Extracellular Tat can act both from the outside of cells and inside of cells. It binds to cellular receptors on the cell membrane triggering different signaling pathways (108). Extracellular Tat may contribute to the spreading of HIV-1 infection by several mechanisms. Secreted Tat is able to transactivate the HIV-1 genome in latently infected cells, thereby increasing the amount of circulating infectious virions (109).

Tat can activate B-lymphocytes and monocytes and make cells of the immune system more susceptible to infection. It also leads to dose-dependent increase in the amount of expression of HIV-1 co-receptors, on the surface of $CD4^{+ve}$ T cells (110). This leads to increased susceptibility to HIV-1 infection (111). Studies using immobilized Tat

demonstrated that the up-regulation of CXCR4 in T cells was truly an extracellular effect of Tat (110).

Tat is shown to be potent chemo attractant that promotes migration of several cell types, including monocytes, endothelial cells, B cells, dendritic cells and polymorphonuclear leucocytes thereby favoring the spread of HIV-1 (112). Tat has sequence homology with β -chemokine and mimics their functional features by signaling through chemokine receptors. This helps in recruiting monocytes and macrophages towards HIV producing cells, which express these receptors (113). These chemotactic properties require both the RGD and the basic domain of Tat (114). These domains are also involved in the migration of dendritic cells and contain only partial activity in induction of monocyte migration (114). A novel region in the cysteine-rich and core domains of Tat mediates the majority of Tat-induced effects on monocytes (115).

Immuno-suppression and apoptosis of uninfected cells seem to be an important cause for the progression of AIDS and Tat plays a role in it. Both Tat and interferon-γ promote the generation of suppressor T cells in HIV-1-infected peripheral blood mononuclear cells (116). In addition, Tat directly inhibits antigen and mitogen induced lymphocyte proliferation (117, 118). Tat also blocks L-type calcium channels, which contributes to progressive immuno-suppression during HIV-1 infection (119). This leads to inhibition of the natural immunity mediated by natural killer cells.

Kaposi's sarcoma is a proliferative disease of vascular origin characterized by proliferating spindle-shaped cells, angiogenesis and inflammatory cell infiltration, which is frequently observed in HIV-1 infected individuals (120) Extracellular Tat promotes the growth of spindle cells derived from AIDS-Kaposi's sarcoma lesions, mediated by the RGD

sequence of the protein (121-123). This indicates that Tat contributes to the development of AIDS-Kaposi's sarcoma.

Tat interaction with cell surface receptors and the consequent activation of the respective intracellular signaling pathways leads to many functions. But several activities of extracellular Tat are mediated by rapid internalization through its basic domain (124). Internalization of full length Tat depends on its interaction with cell membrane heparin sulfate proteoglycans followed by active endocytosis (125).

Most of the effects of extracellular Tat have been observed in tissue culture cells exposed to recombinant Tat. Several investigators have demonstrated that the concentration of Tat to obtain these effects was similar to the concentrations found in HIV-1-infected individuals (111-113, 126).

1.6.15.4 Role of Tat in apoptosis

Conflicting data on the role of Tat in apoptosis has been reported in literature. Tat exerts detrimental effects on a variety of cells including those of immune system. It has been suggested that Tat may play an important role as a secreted, soluble neurotoxin in HIV-1 associated dementia (127). Tat is shown to be actively released into the extracellular space by infected macrophages and glial cells, and may then directly interact with neurons (128-129). Furthermore, Tat appears to induce HIV-1-infected macrophages and microglia to release potentially neurotoxic substances (108). Tat mediates the dysregulation of Caspase 8 pathway which induces apoptosis in neurons and T cells (130). Also, Tat upregulates TRAIL, interacts with cell surface receptor like CD26 and down regulates anti-apoptotic Bcl2, which promotes induction of apoptosis(131-133). Tat also deregulates the

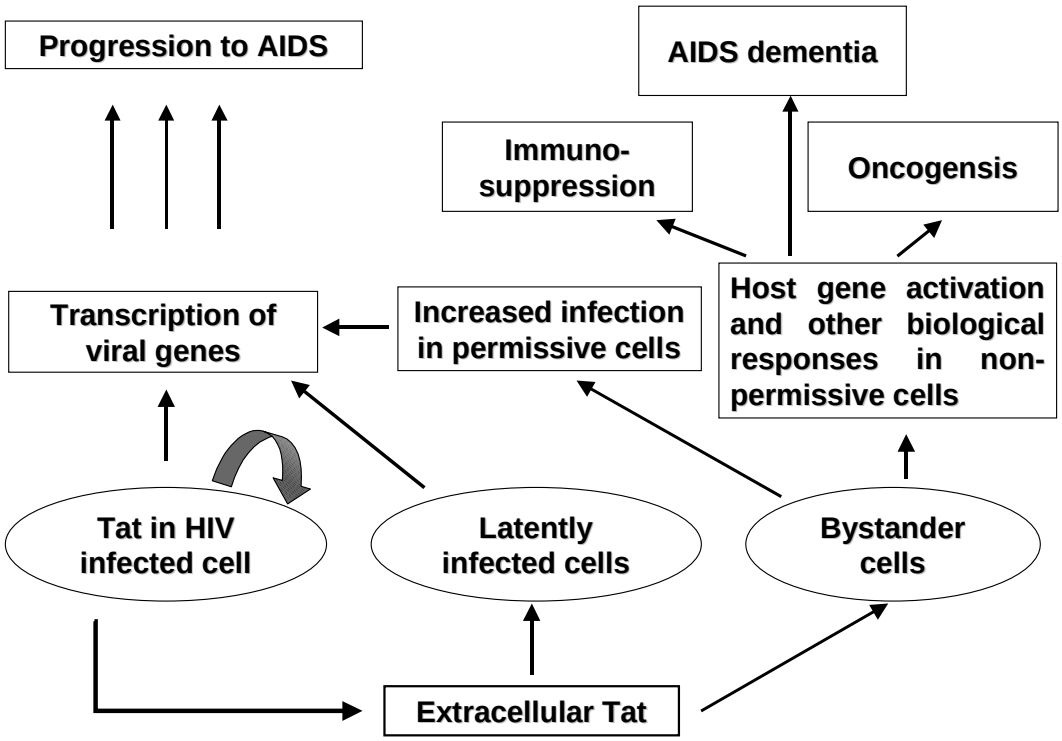


Figure 10: Molecular activities of Tat in HIV-1 infection. (See text for details)

expression of MnSOD, and thus sensitizes the infected cells to lethal effects of reactive oxygen species (ROS) produced by mitochondrial apoptotic pathway (134, 135).

The literature cited above show role of Tat as pro-apoptotic factor. Albeit, there are reports which indicate that it can also act as an anti-apoptotic factor which aids in cell survival. Tat has also been shown to upregulate IL-2 (136) and many other cell survival growth factors including anti-apoptotic genes in infected cells, and hence can protect them against apoptosis (137, 138) The different effects on apoptosis elicited by Tat may depend not only on a differential activation of pro- and anti-apoptotic genes and the cell type involved but also on cytokines and growth factors released in the cell microenvironment. It is interesting to note that in certain neuronal cell line endogenous expression of *tat* gene by transfection did not cause cell death. But when the same cells were exposed to extracellular

Tat they showed significant toxicity. However, Tat-induced neurotoxicity was not observed in all neuronal cell cultures (139). Thus role of Tat in apoptosis remains debatable.

1.7 Aims and Objectives

HIV-1 is the causative agent of AIDS. The expression of the viral genome, which encodes for various regulatory, structural, and accessory proteins, is modulated by regulation of viral LTR promoter. Preceding sections summarizes functional role of HIV-1 proteins in viral replication. Tat protein is the most important regulator of viral gene expression which activates LTR promoter directed transcription by interacting with TAR RNA and recruiting pTEFb complex. Tat protein acts as a pleiotropic factor for a number of functions both inside and outside the cell and plays an important role in viral life cycle. Tat through trans-cellular communication reprograms cellular gene expression of infected as well as uninfected cells and may contribute to wide range of clinical complications. Tat is involved in both initiation and elongation of transcription and can also induce chromatin remodeling by recruiting histone acetyl transferases (HATs). In addition, several studies have shown convincing evidences that Tat can transactivate HIV-1 LTR mediated gene expression in absence of TAR and regulatory elements in the enhancer region of LTR play an important role in it. TAR independent transactivation by Tat is a widely accepted phenomenon but the molecular basis of this activation remains to be clearly understood. Studies in this direction could provide insight to not only in a clear understanding of TAR independent transactivation of LTR by Tat but also to some extent illuminate the complex nature of its functional role in AIDS pathogenesis.

A number of earlier studies clearly indicate that Tat could substantially effect transcription when tethered to DNA. There have also been reports establishing functional similarities between Tat and other transcription factors, 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. In addition, Tat is shown to modulate and de-repress the integrated HIV-1 chromatin structure, aiding in activation of transcription. Interestingly, Tat is unable to either activate transcription or induce changes in chromatin structure of integrated promoter lacking both SP1 and NFκB sites even when it is tethered to the HIV-1 core promoter upstream of the TATA box, indicating thereby the importance of this region in LTR driven viral gene expression in infected cells.

Although the previous reports point towards the possibility of a DNA binding activity for Tat, it has not demonstrated experimentally. Earlier investigations could not find any detectable binding between TAR DNA and purified Tat protein (140). The prime objective of this thesis is to identify target DNA sequences bound specifically by Tat protein. The result of the project will have implications in understanding DNA binding property of Tat, modulation of host cellular genes as result of this interaction which may provide basis for design of new therapeutic interventions. In current work it was proposed:

1) To identify DNA targets of physiological importance for Tat protein.
2) To verify the specificity of this Tat:DNA interaction by oligonucleotide library screening.
3) To carry out biophysical studies to characterize the Tat:DNA interaction.

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Chapter 2: Tat as a DNA binding Transactivator

2.1 Introduction

The transactivator protein, Tat of Human Immunodeficiency Virus-1 (HIV-1) activates long terminal repeat (LTR) promoter directed transcription by interacting with a RNA element named transactivation response region or TAR (1, 2). Among the intricacies of Tat: TAR interaction lays an unanswered question of viral gene expression, i.e., the chronology of the synthesis of these two molecules.

Tat is known to play a role in transcription initiation (3). Recent evidence of Tat expression even before integration of the viral genome (4) indicates a possible role of Tat in transcriptional initiation from the integrated provirus. In addition, several studies have shown convincing evidences that Tat can transactivate HIV-1 gene expression in absence of TAR (4-7). It has also been shown that NFκB and SP1 regulatory elements in the enhancer and core region of LTR play an important role in TAR independent transactivation but the molecular basis of this activation remains to be clearly understood (8, 9). Previous studies have shown that combined NFκB and SP1 enhancer deleted virus becomes replication incompetent (10, 11). Furthermore, absolute requirement of NFκB enhancer element was shown for both Tat dependent and independent viral transcription in blood $CD4^{+ve}$ T cells (6).

Tat has been shown to modulate several host cellular genes and make cellular environment amenable for viral replication (12). A number of earlier studies clearly indicate that Tat could substantially effect transcription when tethered to DNA (8, 9). There have also been reports establishing functional similarities between Tat and other transcription factors (13), which enhance the level of gene expression by binding to DNA.

All These studies summarize the complexities associated with the role of Tat protein in viral replication and hence transcription from the HIV-1 LTR.

Tat is shown to modulate and de-repress the integrated HIV-1 chromatin structure, aiding in activation of transcription. Interestingly, Tat is unable to either activate transcription or induce changes in chromatin structure of integrated promoter lacking both SP1 and NFκB sites even when it is tethered to the HIV-1 core promoter upstream of the TATA box. (14), indicating thereby the importance of this region in LTR driven viral gene expression in infected cells. While, NFκB fails to stimulate the integrated transcriptionally silent HIV-1 promoter (14, 15).

Based on these reports, studies were initiated looking into the molecular mechanism of Tat function in absence of TAR. We asked whether Tat could interact with DNA, specifically with upstream enhancer sequences in LTR as it has been found to be very important for both TAR independent and dependent Tat responsive transactivation of HIV-1 LTR. In order to verify DNA and Tat protein interaction independently, various screening techniques like gel shift assay, reporter gene activation, and SELEX technology have been used.

2.2 Materials and Methods

2.2.1 Oligonucleotides

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. Where required complementary strands of oligonucleotides were annealed to generate double-stranded oligonucleotides by heating equimolar amounts at 94°C for 2 min and subsequently gradually cooling to room temperature in a water bath.

2.2.2 Plasmids

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, with selection marker for *neomycin* resistance, has been described previously (16). An enhanced green fluorescent protein encoding vector pEGFP-N1 was obtained from Clontech (USA).

2.2.3 Tat protein

Recombinant pure Tat was obtained From NIH AIDS Reagent Program (17) or purified from *E. coli* BL21-DE3 transformed with either expression vector GST-Tat1-86R TK or GST-Tat1-86R C22G mutant as reported (18), with minor modifications. The bacterial culture was grown at 37°C to 0.3 OD_{595nm} and induced with 0.1 mM isopropyl β-D thiogalactoside and further grown to 0.6 OD_{595nm}. The bacterial pellet after centrifugation of culture was lysed in 50mM Tris-HCl pH8.0, 5 mM DTT, 1x protease inhibitor cocktail, 0.5 % NP40, 120 mM NaCl. The lysate was incubated with Glutathione-Sepharose beads to allow binding of GST-Tat protein. 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 or eluted with 10mM Glutathione in 50mM Tris-HCl pH 8.0, 5mM DTT and 120mM NaCl. The Gst-Tat protein was dialyzed extensively against 10 mM HEPES, pH

7.9, 50 mM KCl, 5 mM DTT buffer for three times. SDS-PAGE and western blot with polyclonal anti-Tat antibody (19) was used to confirm the purity of both Gst-Tat and recombinant Tat protein.

2.2.4 Cell cultures

HEK 293T and Jurkat (J6) T cell line was obtained from Cell Repository, National Centre for Cell Science, India. CEM-GFP cell line was obtained from NIH AIDS repository (20). HEK 293T cells were grown in DMEM (Gibco-BRL, USA); whereas CEM-GFP cells and Jurkat cells were grown in RPMI 1640 (Gibco-BRL, USA) supplemented with 10% Fetal bovine serum (Gibco-BRL, USA) at 37ºC in 5% CO_2. All the cell lines were maintained in active growth phase.

2.2.5 Preparation of nuclear extract

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 (21). Briefly, 2 $x10^7$ cells were lysed in buffer containing 0.1% NP40, 10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, and 0.5 mM DTT. The nuclear pellet was resuspended in a high salt buffer containing 420 mM NaCl, 20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM PMSF, 0.2 mM EDTA, and 25% glycerol and incubated on ice for 15 minutes. The mixture of nuclear protein was then centrifuged for 10 minutes at 21000x g; the supernatant was diluted with 5 volumes of storage buffer (20 mM HEPES, pH 7.9, 100 mM KCl, 0.5 mM PMSF, 0.5 mM DTT, 0.2 mM EDTA, and 20% glycerol) and kept in aliquots at -70ºC.

2.2.6 Gel shift assay

Duplex oligonucleotides (See Table 1) for gel shift were end labeled with [γ-P^{33}] ATP (BRIT, India) and 10U of T4 Polynucleotide Kinase (Invitrogen, USA) using forward reaction buffer according to manufacturer's instruction. Radio labeled probe (0.02 pmoles) was incubated with Tat in 10mM HEPES pH 7.9, 50mM KCl, 1mM DTT, 2 μg/mL poly dI-dC: dI-dC, 330 μg/ml BSA and 10% w/v Glycerol. The binding reaction was carried at 30ºC for 10 min and was subsequently loaded onto 9% native PAGE (acrylamide: bisacrylamide, 50:1) containing 5% glycerol. The gel was electrophoresed in 130 mM Tris, 45 mM boric acid, 2.5 mM EDTA at 150V for 45 min at 4ºC in a Biorad protean II gel electrophoresis system. The gel was dried and exposed to Kodak Biomax film with intensifying screen. (See Box 1 for general introduction to this method)

Table 1: List of oligonucleotides used in gel shift assay

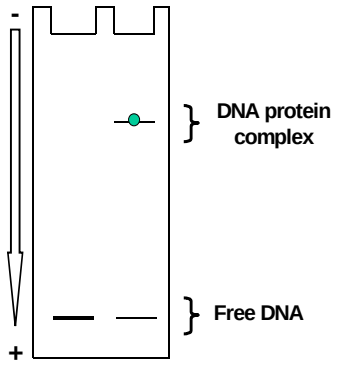
Sr. No.	Sequence	Region (position)*
1	5′ CAAGGGACTTTCCGCTGGGGACTTTCCAGG	NFκB region of HIV-1 LTR (347-376)
2	5′ CAACTCGGTTTCCGCTCTCAGCTTTCCAGG	Mutated NFκB region from HIV-1 LTR
3	5′ AGTTGAGGGGACTTTCCCAGGC	NFκB consensus
4	5′ AGTTGACTCTCAGATGATAGGC	Mutated NFκB consensus
5	5′ ATTCGATCGGGGCGGGGCGAGC	SP1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

* Numbering is based on HIV-1 HXB2 reference sequence

Box 1...

Gel shift assay

The gel mobility shift assay or electrophoretic mobility shift assay (EMSA) has developed into the most widely used method to analyze the binding of proteins to specific sequence of DNA. The value of this assay as a qualitative probe for protein DNA interactions is clearly established. Its popularity is justified by its technical simplicity and by its wide applicability. It is a valuable tool for study of DNA binding proteins present in the nuclear extracts. This method has been used widely in the study of sequence-specific DNA-binding proteins such as transcription factors.



Diagrammatic representation of Gel shift assay-showing retardation in mobility of DNA fragment due to complex formation with protein molecule.

The gel shift assay provides a simple and rapid method for detecting DNA-binding proteins. The assay is based on the observation that complexes of protein and DNA migrate through a non-denaturing polyacrylamide gel more slowly than free DNA fragments or double-stranded oligonucleotides in an electric field. The DNA molecule is highly negatively charged and moves rapidly under the influence of electric field towards the positive electrode. DNA molecules are separated according to their size due to relative ease with which smaller fragments are able to penetrate the fine gel meshwork more easily than large ones. Protein molecules bound to a DNA molecule will cause it to move more slowly through the gel; in general, the larger the size of bound protein, the greater the retardation of the DNA molecule. This phenomenon provides the basis for the gel-mobility shift assay, which allows even trace amounts of a sequence-specific DNA-binding protein to be readily detected. Radiolabelled DNA is used as a probe and proteins samples are incubated with probe to form complex. The reaction products are then analyzed on a non-denaturing polyacrylamide gel.

If the DNA fragment corresponds to a region where, several sequence-specific proteins bind, autoradiography will reveal a series of DNA bands, each retarded to a different extent and representing a distinct DNA-protein complex. The proteins responsible for each band on the gel can then be separated from one another by subsequent fractionations of the nuclear extract.

2.2.7 HIV-1 infection

2 x 10^6 CEM-GFP cells were incubated with 100 ng p24 (equivalent to approximately 0.1 MOI) of HIV-1 NL4.3 (22) viral stock along with 1μg/ml polybrene (Sigma, USA) for 4 hours at 37°C in a humidified incubator with 5% CO_2. These cells were then washed with PBS, transferred to 2 ml of RPMI 1640 with 10% FCS and incubated at 37°C. The CEM-GFP cell line has the Green Fluorescent Protein (GFP) gene stably integrated under the HIV-1 LTR promoter. The progress of infection was visualized by GFP expression and followed by p24 assay of the culture supernatant using p24 antigen ELISA kit (Perkin Elmer life science, USA).

2.2.8 Transfection

2.2.8.1 Transient transfection

HEK 293T cells were plated at density of 6 x 10^5 cells/ well in a six well plate. The cells were allowed to adhere and subsequently plasmid vectors were transfected with Lipofectamine 2000 (Invitrogen, USA) according to manufacturers instructions. Briefly, Lipofectamine 2000 reagent was used at a ratio of 3μL per μg of plasmid DNA. The reagent was diluted in 500 μL of OptiMEM (Invitrogen, USA) and incubated for 15 minutes. The plasmid DNA diluted in OptiMEM (500 μL) was then added dropwise to the diluted lipofectamine reagent. The mixture was incubated for 20 minutes at room temperature to allow formation of lipid and plasmid DNA complex. This was then loaded on to cells which were then incubated at 37°C in a humidified incubator with 5% CO_2. 1 μg of pEGFP-N1 vector (Clontech, USA) was co-transfected in all experiments for normalization based on GFP expression.

2.2.8.2 Stable transfection

Jurkat cells were stably transfected with pCDNA-Tat using electroporation and the cells were incubated with Geneticin (G418 sulfate; Invitrogen, USA) 1000 µg/ml for selecting Tat-expressing Jurkat cells. Reverse transcription PCR and reporter gene transactivation assay using HIV-1 LTR-luciferase vector confirmed the expression of Tat in these Jurkat-Tat cells.

2.2.9 Reporter assay

Cells were harvested 36 hrs post transfection and washed twice with phosphate buffered saline (PBS) 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.4 mM KH_2PO_4 pH 7.3. The cells were then lysed in CCLR buffer (25 mM Tris-phosphate pH 7.8, 2 mM Dithiothreitol, 2 mM 1,2-diaminocyclohexane- N, N, N', N'- tetra acetic acid, 10% Glycerol, and 1% Triton X-100). The lysate was centrifuged at 12000xg for 2 minutes at 4°C and supernatant was used for measurement of both GFP and luciferase activity. All measurements were recorded on Fluoroskan *Ascent* FL micro plate recorder (Labsystem, Finland), which can be used for both fluorescence and luminescence measurements. Prior to estimation of luciferase, the EGFP fluorescence was estimated for the samples in the wells of black combiplate (Labsystems, Finland) with filter set at excitation 485 nm and emission at 510 nm. The luciferase activity was then measured in the same plate using the substrate from single luciferase assay kit (Promega, USA) according to manufacturers' direction. The luciferase activity was normalized to EGFP fluorescence units (signal strength) (23).

Alternatively the assays were carried out using a Luclite™ assay kit (Perkin Elmer Lifescience, USA) and luminescence measurements were carried on TopCount microplate

Box 2...

Transient transfection and Reporter assay

Introduction of foreign genes into mammalian cells or transfection is of great interest both for basic biological research and gene therapy and is routinely practiced in molecular and cell biology laboratories. Transfection of cultured mammalian cells is also a common model system in gene therapy applications to develop DNA delivery systems. Regulatory functions of promoters are normally characterized by transient transfection based reporter gene assay, which form excellent experimental model for such study. The reporter gene is usually placed under the control regulatory unit (promoter) under investigation. These are then put inside cells by various means available. The most popular methods are calcium phosphate precipitation, diethyl amino ethyl dextran co-precipitation, electroporation, dendrimers, and lipofection. Majority of the recent commercial reagents, which provide excellent efficiency of transfection, are lipid-based formulations. One could then study the regulation of expression of this reporter under various experimental conditions. There are a number of *in vitro* reporter genes, such as secreted alkaline phosphatase [SEAP], β-galactosidase [β-gal], Firefly luciferase, and chloramphenicol acetyltransferase [CAT], available for use in these studies (24). A number of methods have been described that utilize simultaneous transfection of second reporter vector as an internal control plasmid (24) to normalize variation in transfection efficiency.

counter (Packard Bioscience, USA). The cells were lysed in CCLR; the supernatant was used to quantitate EGFP on Fluoroskan *Ascent* FL micro plate recorder in the wells of Opti-plate (Packard Bioscience, USA). Subsequently to the same well 100 μL of Luclite reagent was added and luminescence was measured in TopCount. (See Box 2)

2.2.10 Oligo library screening

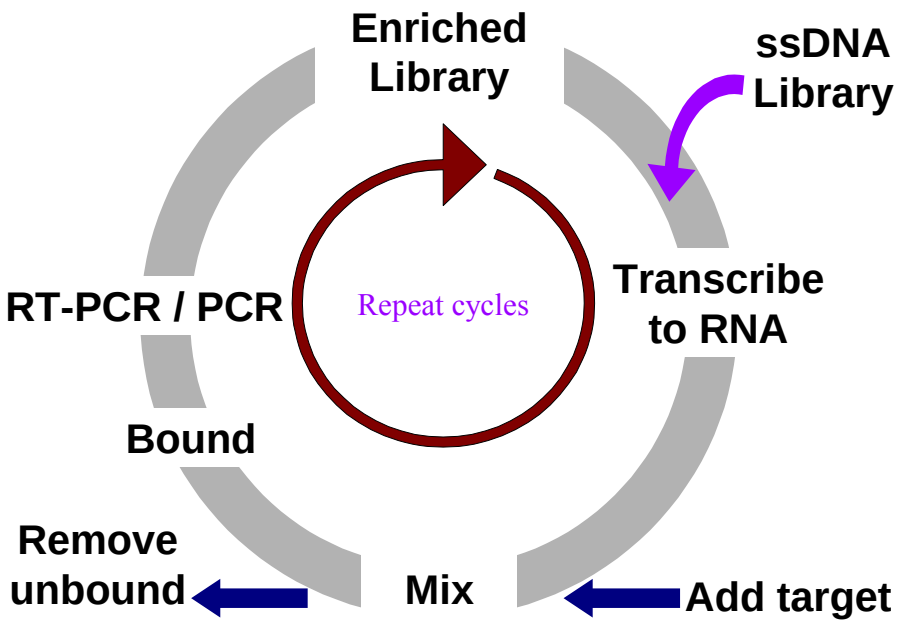
The synthetic random oligonucleotide library (DDSEL) with two fixed primer regions DDCI (sequence: 5′ TCATAAGTAGCGGAGGACGAGACG) and DDCII (sequence: 5′ CGCAGCTACAGTGCAGACAGTCAG) and a central random 32-base region was synthesized using Pharmacia Gene Assembler plus automated synthesizer (Pharmacia, USA). 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 *E. coli* Klenow fragment (Roche, USA) according to the manufacturer's instruction.

Box 3…

Oligo library screening or SELEX

Systematic ***E***volution of ***L***igands by ***EX***ponential enrichment (**SELEX**) also known as *in vitro* selection (25) is a very promising new trend in combinatorial chemistry. The method was simultaneously and independently developed in the laboratories of G. F. Joyce (26), J. W. Szostak (27), and L. Gold (28). *In vitro* selection involves screening of large random pools of nucleic acids for enrichment of molecules with desired properties.



General scheme for SELEX experiment: A library of random DNA sequences generally representing ~10^{15} sequence is used. This is then used directly or transcribed into RNA, purified and allowed to fold into respective structure. The target molecule and library is then incubated together to facilitate binding. A subsequent separation step ensures removal of non-interacting unbound species. The bound sequences are amplified by polymerase chain reaction (PCR). The cycle is repeated several times till sufficient enrichment is achieved.

BOX 3 continued...

Strategy:

The method is simple and easily adaptable. The library or the starting pool is generated on DNA synthesizer. The central random region is flanked by a defined sequence. In this way, up to 10^{15} different DNA molecules can be synthesized at once. SELEX is based on the assumption that library contains a few molecules with the correct receptor structure or with tertiary structures, which will lead to selection of desired property. The ligands are generally selected, by affinity chromatography or filter binding. A very small fraction of functional molecules is present in library and several rounds of selection are usually required to isolate them. Therefore, the very rare active molecules are amplified by the polymerase chain reaction (PCR) or in a transcription-based step. In this way, iterative cycles of selection can be carried out. Successive selection and amplification cycles result in an exponential increase in the abundance of functional sequences, until they dominate the population as depicted in the schematic.

Use:

The method has been applied to a number of different applications; for example, *in vitro* selection has proven to be extremely efficient for the identification of bases critical for the function of ribozymes, or in a protein-binding site in a (ds or ss) DNA or RNA molecule (28). Recently, *in vitro* selection has been used for the *de novo* isolation of catalytic RNAs (29). These include ribozymes with ligation activity, isomerases and ribozymes that catalyze the ATP-dependent phosphorylation of RNA oligonucleotides. Several RNA- and DNA-aptamers have been isolated, which not only bind tightly to proteins, but also are able to inhibit their biological activity (29).

The selection of bound oligonucleotides 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 radiolabelled during amplification by PCR (30 cycles: 95°C for 1 min, 72°C for 1 min) 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. Several positive clones were selected and sequenced. (See Box 3)

2.2.11 Chromatin Immunoprecipitation (ChIP) Assay

The ChIP assay was done as described previously (30, 31) with minor modifications. Briefly, HIV-1 NL4.3-infected CEM-GFP cells were cross-linked for 10 minutes by 1% formaldehyde followed by quenching with 125 mM glycine. Cells were washed with buffer 1 [0.25% Triton X 100, 10 mM EDTA, 10 mM HEPES pH7.5, 1 mM PMSF, 10 mM sodium butyrate, 1x protease inhibitor cocktail (Roche)] and buffer 2 [0.2 mM NaCl, 1 mM EDTA, 0.5mM EGTA, 10 mM HEPES pH 7.5, 1 mM PMSF, 10 mM sodium butyrate, 1x protease inhibitor cocktail (Roche)]. Subsequently 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, 1x 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 NaHCO3 and 10 mM DTT. Cross-links were reversed by addition of 0.10 volumes of 2 M NaCl and incubation for 4 hours at 65°C. The protein from the complex was subsequently digested with proteinase K (100 μg/ml) in presence of 12.5 mM EDTA pH 8.0 and 50 mM Tris-HCl (pH 6.8). DNA was recovered by phenol-chloroform extraction followed by ethanol precipitation (2.5 volumes) in presence of 300 mM sodium acetate (pH 5.2) and 20 μg of glycogen. 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.

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)

* Numbering is based on HIV-1 HXB2 reference sequence

2.3 Results

Although the previous reports point towards the possibility of a DNA binding activity for Tat (8, 9, 13), it has not been verified experimentally. An alignment search for Tat sequence against super families of protein, based on structural classification of proteins using Hidden Markov Model, (32) with software Superfamily 1.61 (http://www.supfam.org) was performed to determine the homology with known DNA binding proteins. The examination of HIV-1 Tat sequence suggested that it contained structural motifs homologous to N terminal domain of mouse and human NFκB p50 subunit (Figure-1) and it belongs to p53 super-family of transcription factors. This similarity in structural motif could be due to the presence of structurally conserved amino acid residues like Val (4^{th}), Cys (22^{nd}) Leu-Gly-Iso (43^{rd} - 45^{th}), and Lys-Lys (50-51^{st}).

Based on previous reports and indication obtained from our structural alignment results mentioned above, gel shift assays were performed to identify DNA binding activity of purified recombinant Tat protein with NFκB enhancer sequences (Table-1). Recombinant

Tat protein was over expressed in *E. coli* BL21-DE3 and purified using Glutathione Sepharose affinity purification. The purity of protein was confirmed by SDS-PAGE and western blot (Figure-2). As shown in Figure-3, 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 super shift using Tat antibody (Figure 3A, lane 6).

```
     1         10         20         30         40         50
     |         |          |          |          |          |
Tat  MEPVDPRLE.PWKHPGSQPKTA.CTNCYCKKCCFHCQVCFITKALGISYGRKKRR
1nfk VQLVTNGKN.IHLHAHSLVGKH.CEDGVCTVTAGPKDMVVGFANLGILHVTKKKV
1svc VQLVTNGKN.IHLHAHSLVGKH.CEDGICTVTAGPKDMVVGFANLGILHVTKKKV
1gji TTLVTKNEP.YKPHPHDLVGKD.CRDGYYEAEFGPERRVLSFQNLGIQCVKKKDL
2ram ISLVTKDPP.HRPHPHELVGKD.CRDGYYEADLCPDRSIHSFQNLGIQCVKKRDL
1ikn ISLVTKDPP.HRPHPHELVGKD.CRDGYYEADLCPDRSIHSFQNLGIQCVKKRDL
1nfi ISLVTKDPP.HRPHPHELVGKD.CRDGFYEAELCPDRCIHSFQNLGIQCVKKRDL
1vkx ISLVTKDPP.HRPHPHELVGKD.CRDGYYEADLCPDRSIHSFQNLGIQCVKKRDL
1bvo VSCVTKEGPeHKPHPHNLVGKEgCKKGVCTVEINSTTMSYTFNNLGIQCVKKKDV
         60         70         80         90         100        110
         |          |          |          |          |          |
Tat  Q----------RRRAHQNSQTHQASLSKQPTSQPRGDPTGPKEKKKVERETETDP
1nfk FETLEARMTEACIRGYNPGLLVHSDLAYLQAEGGGDRQLTDREKEIIRQAAVQQT
1svc FETLEARMTEACIRGYNPGLLVHPDLAYLQAEGGGDRQLGDREKELIRQAALQQT
1gji KESISLRI-SKKINPFN----------------------------VPEEQLHNI
2ram EQAISQRI-QTNNNPFHVPI-------------------------------EEQR
1ikn EQAISQRI-QTNNNPFHVPI-------------------------------EEQR
1nfi EQAISQRI-QTNNNPFQVPI-------------------------------EEQR
1vkx EQAISQRI-QTNNNPFHVPI-------------------------------EEQR
1bvo EEALRLRQ-EIRVDPFRTGFGH----------------------------AKEP
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Figure 1: Multiple alignment of sequence obtained from SCOP database searches with Superfamily, version 1.61. Tat protein aligns with p53-like transcription factor super-family with maximal homology to N-terminal domain of mouse and human NFκB p50 (model no-0003904, Expect value: 2.9e -0.1). The proteins are identified by PDB entry codes on the left hand side of the alignment. 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).

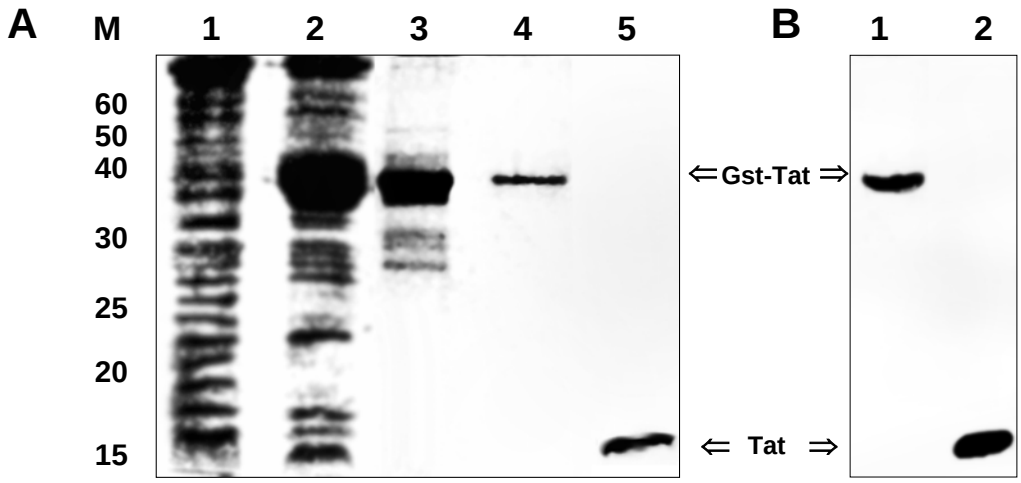

Figure 2: SDS-PAGE and Western blot of recombinant proteins used for binding studies. **A**) SDS-PAGE. M: Marker, Lane 1: Lysate, Lane 2: supernatant, Lane 3: GST-Tat bound to beads, Lane 4: GST-Tat, Lane 5: Tat; **B**) Western Blot of protein with anti-Tat antibody followed by chemiluminescent detection. Lane 1: GST-Tat, Lane 2: Tat.

Gel shift experiments were also performed with the oligonucleotide sequence present in the HIV-1 LTR representing NFκB enhancer sequence (Table-I) for Tat interaction. Again Tat specifically bound the wild type LTR NFκB while no binding was observed with a mutated oligo (Figure-3B). To establish further the specificity of this binding, we performed gel shift assay with two additional enhancer oligonucleotides present in the HIV-1 LTR, AP1 and SP1. Both these sequences did not show any interaction with Tat protein although they interacted with the cellular proteins present in nuclear extract (Figure-3C), demonstrating thereby the specificity of Tat-NFκB enhancer interaction. It is well established that Zinc ions play an important role in self-dimerization and interaction of Tat with other proteins (33, 34). To study the effect of Zinc on Tat-DNA binding, gel shifts were performed in presence of 1.25 mM $ZnCl_2$. Around two-fold enhancement in Tat-NFκB binding was observed in presence of Zn ions. (Figure-3D)

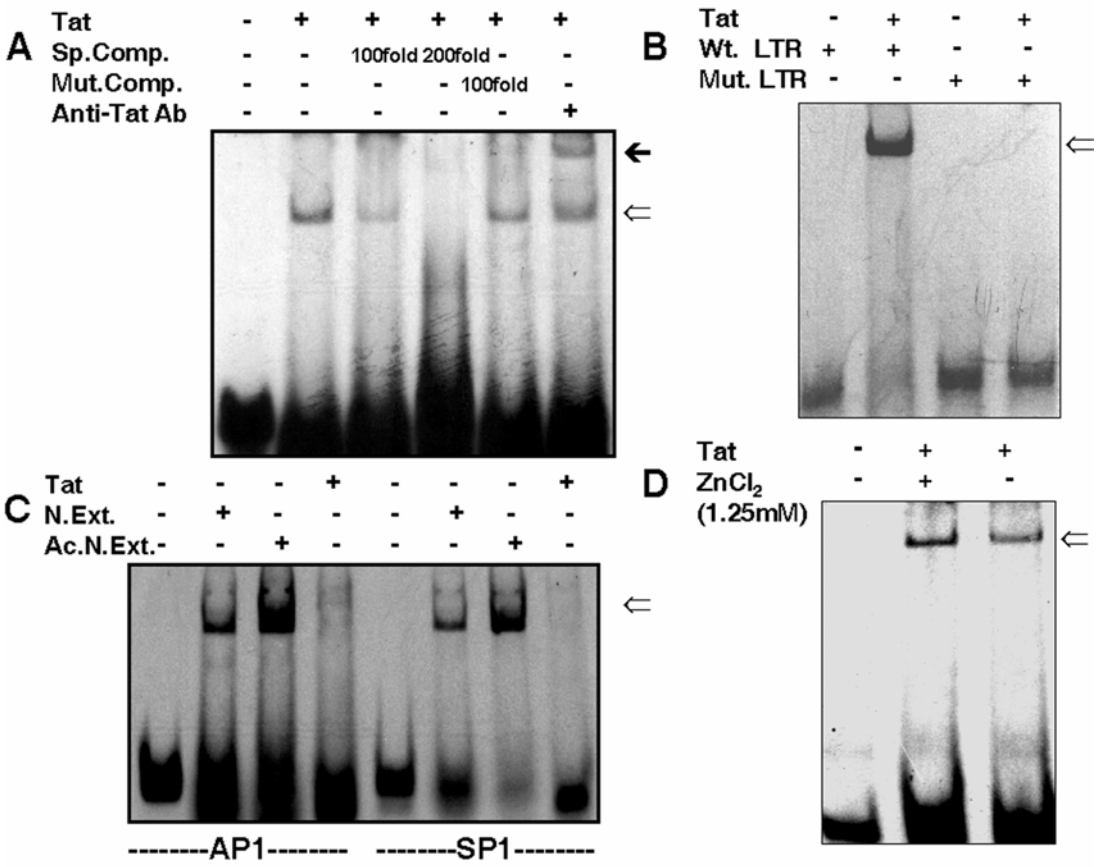

Figure 3: Tat interacts specifically with NFκB enhancer sequences. A) Gel shift assay using NFκB consensus oligonucleotide as labeled probe and cold specific and mutated oligonucleotide as competitors. The sequences are given in Table-I. Lane1: Free probe; Lane 2: Tat protein; Lane 3: 100 fold excess of cold specific oligonucleotide; Lane 4: 200 fold excess of cold specific oligonucleotide; Lane 5: 200 fold excess mutated oligonucleotide; Lane 6: Super shift with anti-Tat antibody. **B)** Gel shift assay using wild type and mutated HIV-1 LTR NFκB oligonucleotides (Table-I) as labeled probe. Lane1: 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-I) with Tat protein and Jurkat nuclear extracts. Lanes 1-4 depicts use of AP1 and 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; and Lane 8: Tat. **D)** Gel shift showing enhancement of Tat binding to HIV-1 LTR NFκB oligonucleotide in presence of 1.25mM $ZnCl_2$. Lane 1: free probe; Lane 2: Tat (500ng) in presence of Zn^{2+} cations; Lane 3, Tat (500ng).

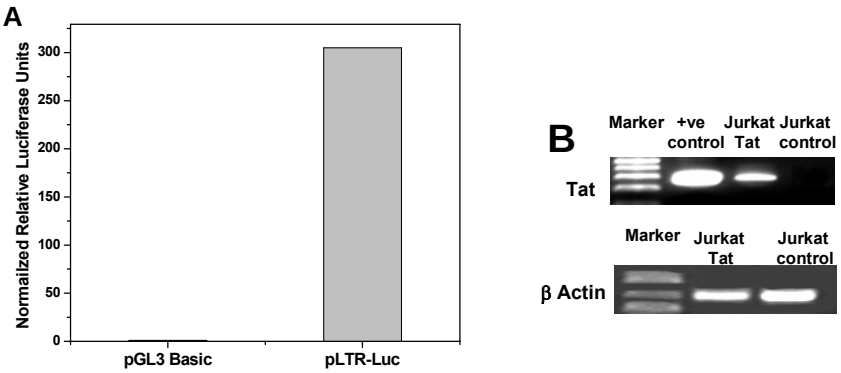

Figure 4: Expression of Tat in Jurkat-Tat cell line. A) Jurkat-Tat cell line was transfected with either pGL3 Basic or HIV-1 LTR-Luciferase construct. Luciferase assays were performed 36 hours post-transfection. The data shown is mean of two independent experiments. **B**) RT-PCR analysis of RNA isolated from Jurkat-Tat to confirm the expression of the Tat gene in cell line (upper panel). Tat plasmid template is used as positive control. β actin is used as loading control(lower panel).

In order to check whether NFκB enhancer sequence could bind to Tat in presence of NFκB protein in nuclear extract, binding studies of LTR-NFκB probe were carried with the nuclear extract prepared from Jurkat-Tat cells stably expressing Tat endogenously. Jurkat cells were initially transfected with pCDNA-Tat and selected in media supplemented with Geneticin sulphate. The expression of Tat protein was confirmed by RT-PCR and transactivation of HIV-1 LTR luciferase reporter construct (Figure-4). The Jurkat-Tat nuclear extract gave shifted complexes with LTR-NFκB probe, which seems to comprise of both Tat and NFκB bound shifted complexes, as is evidenced by super shift with both Tat and p65 (NFκB) antibody (Figure-5 A). In the control experiment only a NFκB mediated shift was observed in un-transfected Jurkat nuclear extract (Figure-5 B). Further gel shift were carried with truncated and mutant Tat protein to confirm this binding. Mutant Tat protein C22G that is deficient in TAR independent transactivation function did not show any binding denoting the specificity of binding (Figure-5 D).

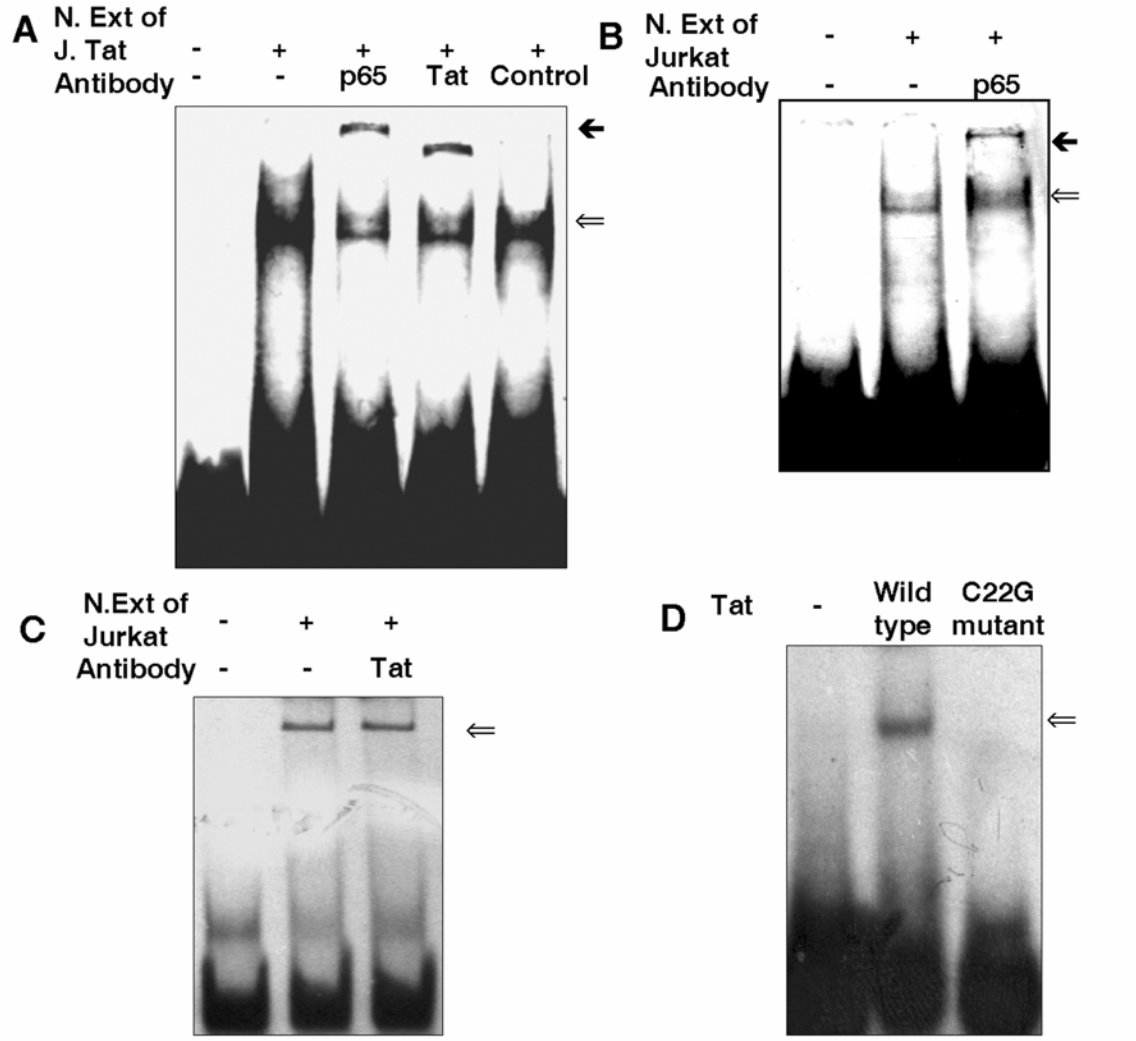

Figure 5: LTR-NFκB enhancer interacts with purified Tat protein and endogenously expressed Tat but not with C22G mutant Tat protein. A) Gel shift assay showing binding of endogenously expressed Tat in Jurkat-Tat nuclear extract to LTR NFκB oligonucleotide. Lane 1: free probe; Lane 2: Nuclear extract of Jurkat-Tat; Lane3: Super-shift with anti-p65 (NFκB) antibody; Lane4: Super-shift with anti-Tat antibody; Lane5: Super-shift with isotype control antibody. Arrows in the figure 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; Lane3: Super-shift with anti-p65 (NFκB) antibody. Arrows in the figure 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; Lane3: Nuclear extract of Jurkat with anti-Tat antibody. Arrow in the figure indicate shifted complex. **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). Arrow in the figure indicate shifted complex.

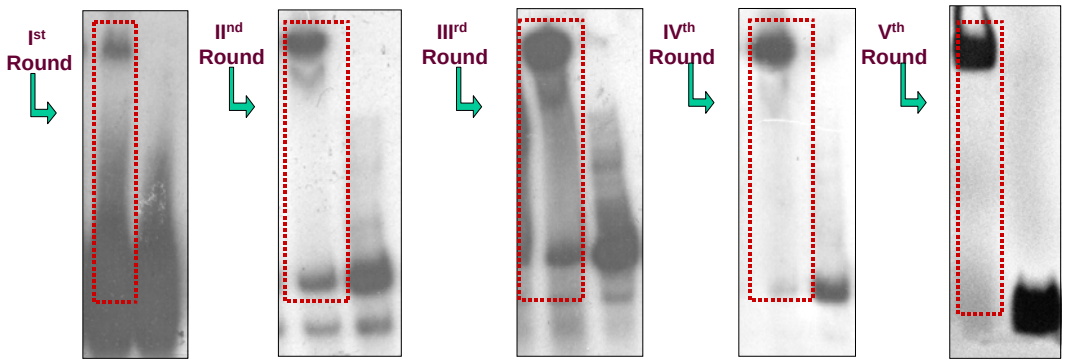

Figure 6: Screening of DDSEL oligo library. The library was PCR amplified and radiolabelled with α ^{32}P dATP during amplification. The amplified library was used for screening for Tat binding DNA sequence by gel shift for five rounds. Every round the bound sequences were gel extracted and reamplified and used in subsequent round.

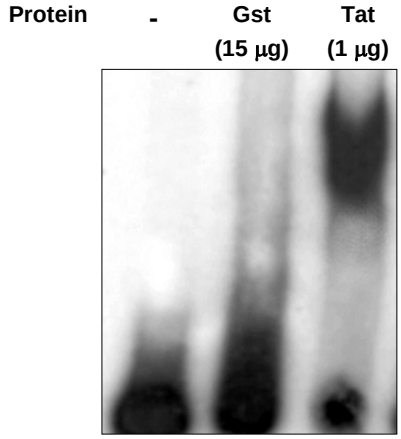

Figure 7: Binding of Tat to pool of sequences selected by library screening. The library selected after five rounds of screening was radiolabelled and used to determine specificity of binding to Tat. Lane 1: Free pool; Lane 2: GST protein (15µg); Lane 3: Tat (1 µg).

To further verify this NFκB enhancer DNA and Tat protein interaction independently, an oligonucleotide library was screened for putative Tat binding sequence using SELEX technology. Iterative screening of oligonucleotide library with GST-Tat protein was carried out to determine consensus-binding motif for Tat protein (Figure-6). The specificity of selected sequences towards Tat was confirmed by gel shift. These sequences were bound only by Tat and not by GST fusion tag of the protein (Figure-7). The sequences internal to the primer in the selected sequences were analyzed using a hidden markov model based motif discovery tool MEME (Multiple EM for Motif Elicitation) software version 3.0

(http://meme.sdsc.edu) (35) to identify 15 base pair long motif bound by Tat protein with the experimentally obtained sequences. The multilevel consensus sequence generated by the matrix corresponds very closely to the complementary NFκB consensus enhancer sequence. (Figure-8)

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 towards the importance of NFκB enhancer sequence of LTR in TAR independent transactivation (4, 5, 8), but the effect of Tat on isolated NFκB enhancer has not been studied. *In vivo* role of the observed Tat- NFκB DNA binding was established by transient transfection studies in

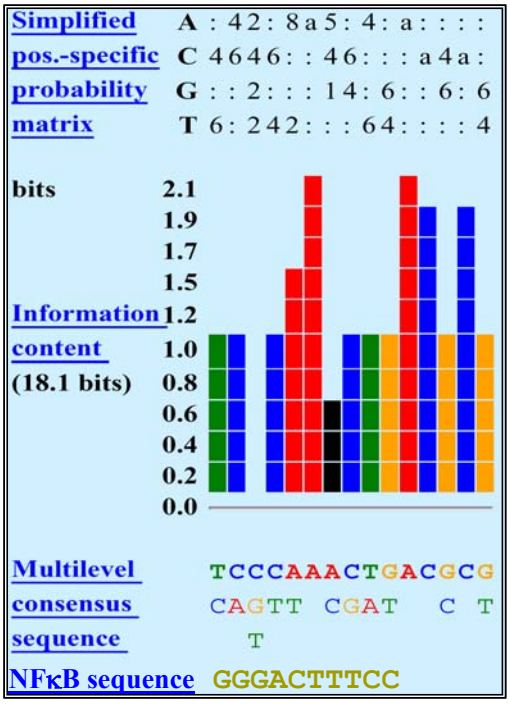

Figure 8: Position specific matrix generated by MEME software analysis. DNA sequences obtained by oligonucleotide library screening were analyzed as described in the text. The matrix shows bit score for occurrence of particular nucleotide in the motif, total bits score as calculated and consensus obtained. The sequence derived is complementary to NFκB enhancer like sequence

HEK 293T cell line. Dose dependent increase in reporter activity (Figure-9) 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 (Figure-9).

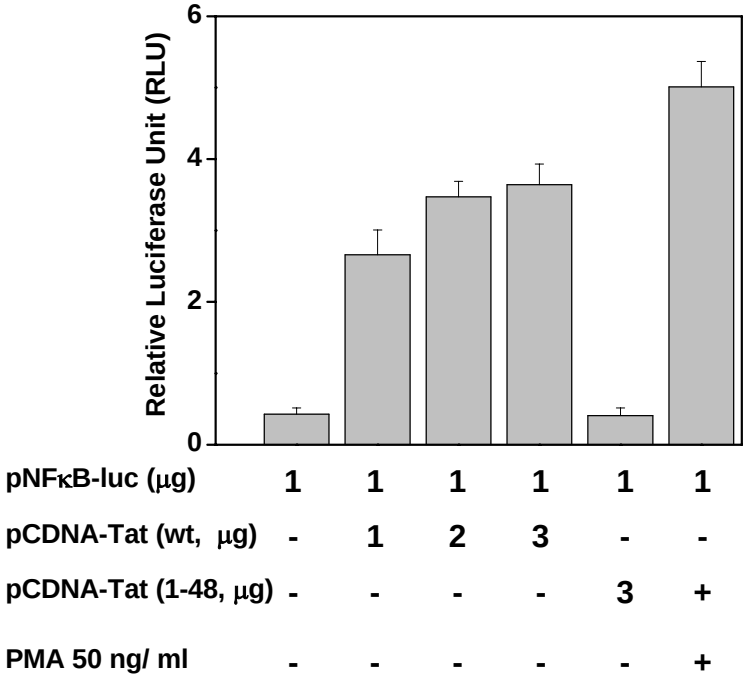

Figure 9: HIV-1 Tat protein activates NFκB enhancer driven reporter gene expression. HEK 293T cells were transfected with pNFκB-luc (1 μg) reporter vector together with either pCDNA-Tat or pCDNA-Tat (1-48), or stimulated with 50 ng/ml Phorbol 12-Myristate 13-Acetate (PMA). The normalized data shown represent the mean and SEM of three independent experiments.

Varying concentration of aspirin (sodium salicylate), a NFκB protein complex specific inhibitor (36), was used in transfection experiments to determine the specificity of Tat mediated transactivation of reporter. Phorbol 12-Myristate 13-Acetate (PMA), a known inducer of NFκB protein complex was used as positive control. A clear decrease was observed in PMA induced enhancement of gene expression. This inhibition effect was

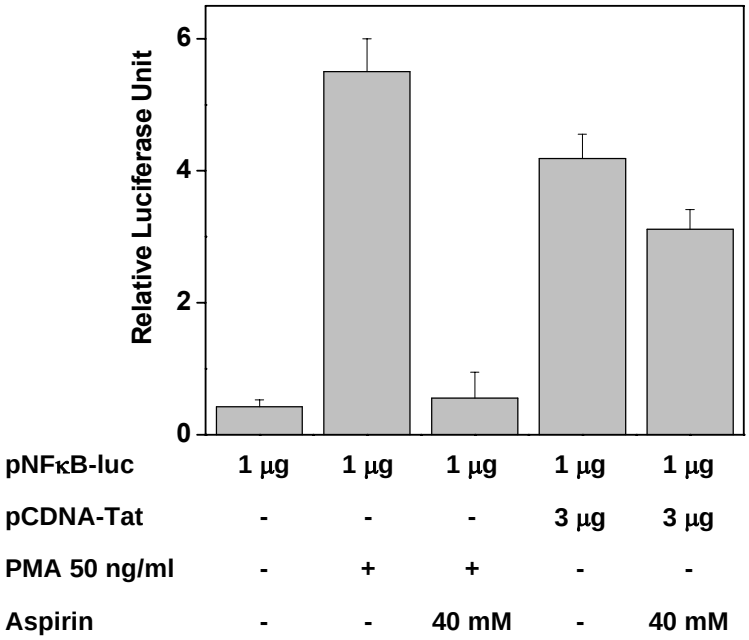

Figure 10: HIV-1 Tat mediated activation of NFκB enhancer driven reporter gene expression is independent of NFκB induction. HEK 293T cells were transfected with pNFκB-luc (1 μg) reporter vector with either pCDNA-Tat or stimulated with 50 ng/ml Phorbol 12-Myristate 13-Acetate (PMA). The cells were then treated with 40 mM aspirin, an inhibitor of NFκB complex. The normalized data shown represent the mean and SEM of three independent experiments.

much reduced in case of Tat mediated activation (Figure-10). Dichloro-1-β-ribofuranosyl benzamidazole (DRB), a Tat activated kinase specific inhibitor (37, 38), completely obliterated the transactivation of pNFκB-luc by Tat (Figure 11) but not by PMA.

In addition to 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 chromatin immunoprecipitation 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-12A represents the cross-linked DNA-protein complex sheared by sonication (input sample) prior to immunoprecipitation. PCR amplification with LTR specific primers (Table-2) flanking 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 (Figure-12 B). To rule out any nonspecific amplification, a control PCR was carried out using HIV-1 nuc-1and 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 (Figure-12 C). This experiment conclusively shows the binding of Tat protein to enhancer region of the LTR integrated in chromatin and supports an important role for Tat in chromatin remodeling as reported earlier (39).

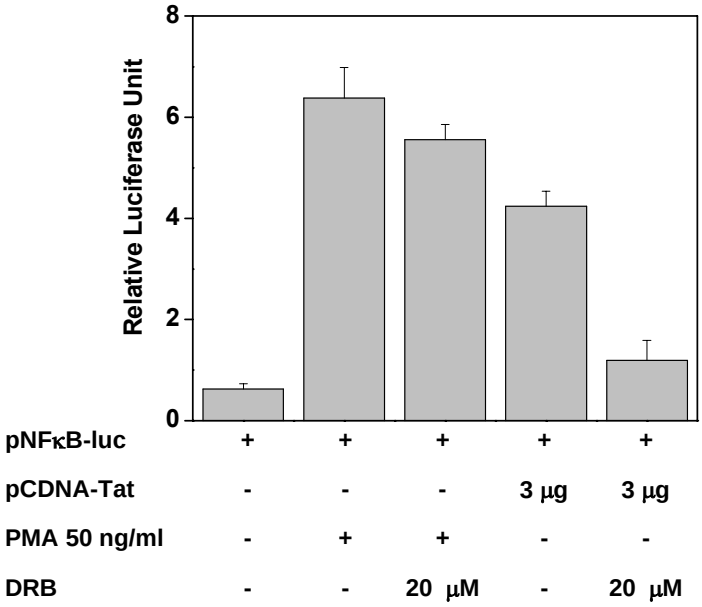

Figure 11: HIV-1 Tat activation of NFκB enhancer driven reporter gene expression is sensitive to Tat activated kinase (TAK) inhibitor. HEK 293T cells were transfected with pNFκB-luc (1 μg) reporter vector with either pCDNA-Tat or stimulated with 50 ng/ml Phorbol 12-Myristate 13-Acetate (PMA). The cells were then treated with 20 μM DRB, an inhibitor of TAK complex. The normalized data shown represent the mean and SEM of three independent experiments.

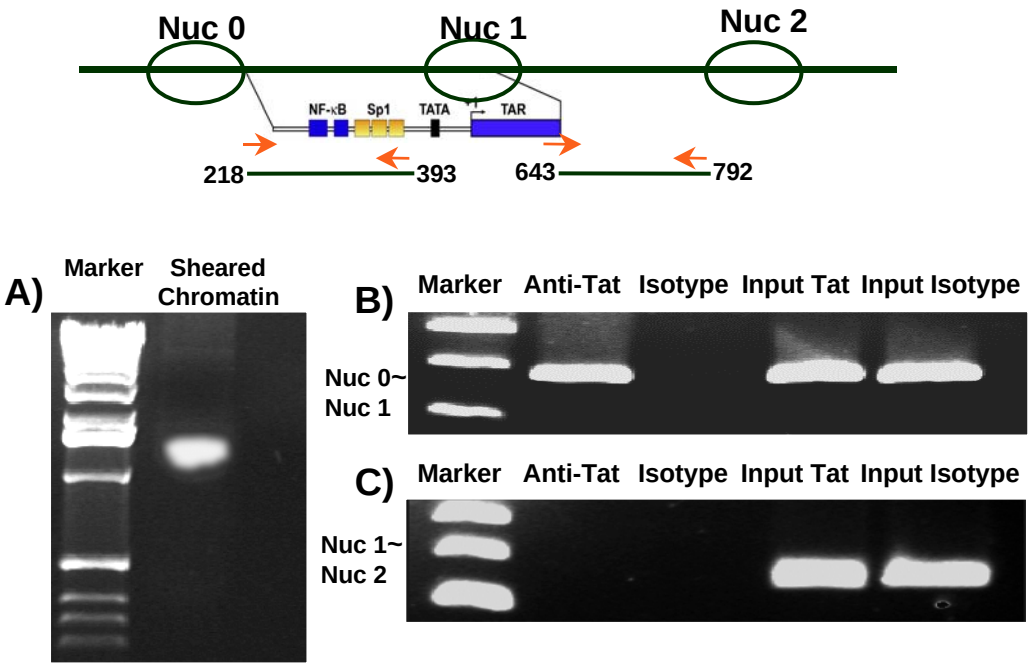

Figure 12: Chromatin immunoprecipitation assay showing interaction of Tat with enhancer region of the integrated LTR *in vivo* in HIV-1 infected CEM-GFP cells. **A)** Chromatin isolated after cross-linking of infected cells was sheared by sonication and ran on agarose gel and visualized by ethidium bromide 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.

2.4 Discussion

Multiple regulatory elements are required for activation of HIV-1 LTR. The activation is dominated by Tat-TAR interaction; however, TAR independent activation has also been widely reported for Tat indicating alternative mechanism for Tat functions (4, 5, 8). In addition to 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 (8, 9, 13). Using Gal4 DNA binding

domain fusion of Rel A and Tat protein, Yang and his coworkers (40) 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 (8). Their data indicates that tethering of Tat on to the enhancer region of LTR containing NFκB and SP1 elements is critical for TAR independent transactivation (40). Also Tat is unable to either activate transcription or induce changes in chromatin structure of integrated proviral promoter lacking both SP1 and NFκB sites (14). However, no direct DNA binding activity has been shown with native Tat protein and proposed cognate target remains to be identified.

An initial structural homology search of Tat protein sequence using Hidden Markov Model indicate that HIV-1 Tat shows maximal homology with mouse and human p50 protein, a member of 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 could also bind specifically to canonical NFκB enhancer sequence pointing thereby to its possible role in modulating cellular gene promoters. Tat has already been shown to modulate expression of several cellular genes (12) in 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 Tat does not bind to canonical SP1 and AP1 sequences which are important elements of LTR mediated gene expression. Also a point mutant Tat C22G failed to bind the NFκB enhancer sequence in the LTR. This also confirmed the definite interaction between Tat and DNA. Furthermore, Tat induced activation of the NFκB enhancer driven

luciferase expression provides functional relevance to this interaction. It could be argued that Tat enhances translocation of NFκB complex thereby inducing the reporter construct. Therefore aspirin, an inhibitor of activation induced NFκB complex, was used in transfection experiments. Almost complete inhibition of PMA induced activation was observed, while the inhibition effect was less in case of Tat mediated activation. This indicates both Tat and to some extent NFκB complex plays a role in induction of gene expression. To delineate the two a Tat specific inhibitor was utilized to provide a definite proof of function. It is well established that Tat activates LTR by recruitment of TAK to the promoter. DRB, a TAK inhibitor was used during transfection studies. Specific inhibition of Tat activation by DRB provides a clear and definite proof for Tat mediated NFκB enhancer dependent reporter gene transactivation. 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). Tat through its multifaceted activity 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 (41-44), which have pleiotropic functions in chromatin modulation and gene regulation. Interestingly, it has been proposed in a recent report (39) that the Tat protein in the monocytic U1 cell line is able to recruit P/CAF to promoter although it lacks its own transactivation function, resulting in 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 (45-47) and also with Cyclin T1 (34, 48) and thus may synergies to enhance the level of transactivation. Recently the role of SP1 in recruiting Cyclin T1 has been elucidated (49). 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 of 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 synergies with Tat and may tether it to the enhancer region of the promoter (44). Our results showing Tat-DNA interaction could be important not only in case of cells of neuronal origin where TAR independent transactivation by Tat has been shown to have profound effect (4, 5, 40, 50), 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 of Tat to chromatin. In light of this new information, it can be proposed 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 of Tat to its binding site on the LTR. Thus Tat binding specifically to chromatin enhancer sequence elements could be the basis for not only TAR independent transactivation of HIV-1 LTR but also modulation of cellular gene promoters. Finally, this interaction adds a new paradigm to increasing list of pleiotropic activities of the Tat protein.

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Chapter 3: Biophysical studies of Tat: DNA interaction

3.1 Introduction

Biochemical studies on individual proteins and their complexes have provided invaluable information on the molecular interactions that can occur within a complex. However, a complete understanding of the molecular basis for their function requires knowledge of their stability, specificity and mechanism of interaction, which can be achieved only through quantitative thermodynamic and kinetic studies.

Tat is a relatively unstable random coil protein with no known structural motifs and no available crystal structure (1). Biophysical and biochemical studies have been used to predict the mode of Tat: TAR interaction employing chemical modifications, cross-linking, affinity cleavage analysis and high-resolution foot printing of Tat: TAR complex (Figure 1). On the basis of the above studies, the proposed model for Tat: TAR recognition consists of the amino terminus of Tat making contact in the wide major groove of TAR RNA (2). This results in conformational change in TAR RNA, which wraps around the Tat peptide, inducing rearrangement and ordering of structure. Based on above studies, it has been

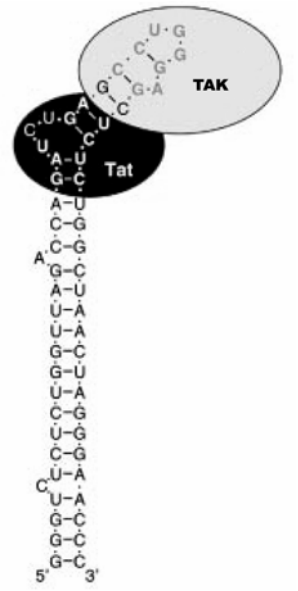


Figure 1: Model for Tat: TAR RNA interaction. TAR RNA folds into the stem loop structure. Tat is able to bind directly to the region near to the apex of the stem containing a UCU bulge. Critical residues for Tat binding are shown in bold. In addition to acting as the binding site for Tat, TAR acts as the recognition signal for a cellular co-factor interacting with its apical loop sequence "Tat activated kinase (TAK)" (Modified from Karn, J. (1999) Tackling Tat. *J. Mol. Biol.,* **293**: 235-254)

suggested that neither the protein nor RNA has the correct structure before there interaction (3). Although the integrity of cysteine region of Tat appears to be essential for its function it is dispensable for TAR binding.

Earlier chapter demonstrated a specific and functional binding of Tat with NFκB enhancer element both *in vitro and in vivo*. In order to elucidate on how Tat exerts its TAR independent function it is necessary to further characterize the Tat: NFκB enhancer DNA interaction. Studies were initiated on characterization of this interaction using various biophysical techniques to determine the strength and nature of binding. The structural rearrangement of protein upon binding to DNA was studied by circular dichroism spectroscopy (CD). These studies were followed by filter binding assay, which was used for determination of equilibrium binding constants. Fluorescence spectroscopy has been proved to be a powerful technique for investigating the interaction of protein with various ligand and, notably nucleic acids (4, 5). In the present study, fluorescence anisotropy has been used to examine the specificity and affinity of Tat: NFκB DNA interaction under equilibrium binding conditions. Furthermore, isothermal titration calorimetry (ITC) is used to determine the kinetics and heat changes associated with the binding of Tat protein to NFκB DNA.

3.2 MATERIALS AND METHODS

3.2.1 Purification of Tat protein

Recombinant GST-Tat and native Tat proteins were purified from *E. coli* BL21-DE3 transformed with expression vector GST-Tat 1-86R TK, or GST-Tat 1-86 R C22G (6) as described in chapter 2, section 2.2.3.

3.2.2 Oligonucleotides

Oligonucleotides (Table 1) either unlabeled or labeled with Fluorescein at 5′ end were obtained by custom synthesis from Genomechanix, USA. The oligonucleotides were purified by two round of reverse phase high-pressure liquid chromatography to ensure maximal homogeneity. Complementary strands of oligonucleotides were annealed to generate double-stranded oligonucleotides by heating equimolar amounts at 94°C for 2 min and subsequently gradually cooling to room temperature in a water bath.

Table 1 List of oligonucleotides used in DNA binding studies

Sequence No.	Sequence	Description
1	5' CAAGGGACTTTCCGCTGGGGACTTTCCAGG 3'	**NFκB region of HIV-1 LTR***
2	5' CAACTCGGTTTCCGCTCTCAGCTTTCCAGG 3'	**Mutated NFκB region of LTR***
3	5' AGTTGAGGGGACTTTCCCAGGC 3'	**NFκB Consensus***
4	5' AGTTGACTCTCAGATGATAGGC 3'	**Mutant NFκB***
5	5' **Fluorescein** AGTTGAGGGGACTTTCCCAGGC 3'	**NFκB Consensus**
6	5' **Fluorescein** AGTTGACTCTCAGATGATAGGC 3'	**Mutant NFκB**

*Same oligonucleotides sequences were used for gel shift assays in Chapter 2

3.2.3 Gel Shift Assay

Gel shift assays were performed as described in chapter 2 section 2.2.6.

3.2.4 Circular Dichroism

Circular dichroism spectra were collected on Jasco-715 spectropolarimeter, using 1 nm bandwidth. CD spectra were averaged for ten accumulations at scan speed for 100 nm/min to improve signal to noise ratio. A 0.10 cm path length quartz cell was used for all measurements. All spectra were collected by dilution of the protein and oligonucleotides in phosphate buffered saline (pH 7.4).

3.2.5 Filter binding assay

Tat protein (1μM) was incubated with varying concentrations of radiolabelled double stranded NFκB oligonucleotide. The binding reactions were carried out in 10 mM HEPES, pH 7.9, 50 mM KCl, 5 mM DTT, 330 μg/mL BSA and 10% w/v Glycerol at 25ºC for 1Hrs. The reaction mixture was loaded on a MF-Millipore Membrane Filter (HAWP01300) pre-wetted with binding buffer. The filter was subsequently washed twice with binding buffer to remove the unbound oligonucleotide. The filters were air-dried and the amount of bound radioactivity was estimated by scintillation counting in a TopCount microplate counter (Packard, USA). The dissociation constant (K_d) was estimated by saturation binding.

3.2.6 Fluorescence Anisotropy Assays

Concentrated stocks (3.5 μM) of double stranded oligonucleotides were diluted to the 3.5 nM in fluorescence anisotropy buffer (10 mM HEPES pH 7.9, 5 mM DTT, 330 μg/mL BSA). The fluorescein-labeled oligonucleotide was titrated with increasing amounts of protein. The samples were incubated in a temperature-controlled cell for 15 minutes at

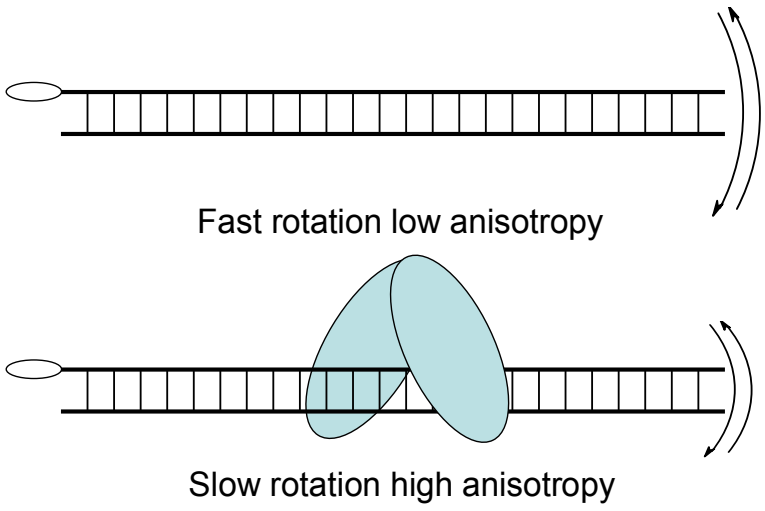
30°C to allow protein-DNA complex formation. Longer incubations did not increase the anisotropy values observed for the Tat: DNA complex, indicating that the reactions had reached equilibrium conditions. Fluorescence anisotropy and total intensity were measured for each dilution using Perkin Elmer luminescence spectrometer LS50B. Samples were excited at 480 nm polarized light and parallel and perpendicular emission intensities were measured at 514 nm. Fluorescence anisotropy was typically calculated from three to four sets of emission measurements, and the average values were used for all further calculations. The standard deviation for the anisotropy values was 0.001 or less. The data was analyzed with Dynafit Software (version. 3.28.024; Biokin Ltd, USA) (7). (See Box 1)

Box 1
Fluorescence anisotropy

The function of almost all proteins is related to their ability to bind ligands. The binding of a ligand to a receptor can be followed by monitoring changes in structure/ conformation of ligand or the receptor. Fluorescence properties of a protein molecule like change in emission and excitation wavelength, intensity or anisotropy can be utilized for these studies. Fluorescence spectroscopy involves the detection of the light emitted by a fluorophore following its excitation with light of a wavelength at which fluorophore absorbs maximum. The advantage of fluorescence based assays over other existing methods is that these assays are carried out in solution, without the need for separation and quantitation of bound and unbound species. Another powerful aspect of fluorescence approach is sensitivity and dynamic range of working concentrations. This combination of sensitivity and versatility along with ease of use makes fluorescence ideal for characterization of complex structure and dynamics, as well as more routine characterization of affinities.

One of the most useful fluorescence properties which can be utilized for monitoring complexation is fluorescence anisotropy/polarization. When a fluorophore is immobile, and the excitation light is polarized, the emitted light will also exhibit the same polarization. In contrast, when a fluorophore is sufficiently mobile, the orientation of the fluorophore is partially or completely randomized and the emitted light will exhibit lesser polarization.

Box 1 continued...



Schematic representation of the effect of protein binding on anisotropy of a fluorescently labeled oligonucleotide.

Due to different hydrodynamic properties, a high molecular weight species normally rotates in solution more slowly than a low molecular weight ligand species. Therefore, on excitation with polarized light a higher molecular weight species will normally emit light with a greater polarization than a lower molecular weight species. This dependence of emitted light polarization on the size of the fluorophore and fluorophore macromolecule complexes, allows useful application of fluorescence polarization/anisotropy measurements for studies of macromolecular interactions.

Measurement of fluorescence polarization/anisotropy involves the excitation of a sample with polarized light and determination of the extent of polarization of the emitted light. The results of a measurement can be expressed in terms of fluorescence polarization (P).

$$P = (I_{\parallel} - I_{\perp}) / (I_{\parallel} + I_{\perp})$$

Where $I_{\parallel}$ and $I_{\perp}$ are the intensities of the emitted light parallel and perpendicular to the plane of polarization for excitation light.

Anisotropy (r) on the other hand is defined as

$$r = (I_{\parallel} - I_{\perp}) / (I_{\parallel} + 2I_{\perp})$$

It is preferable to employ anisotropy rather than polarization because the former is normalized to total intensity. It is therefore a sum of the anisotropies of the individual species present in solution weighted for their fractional populations and if necessary relative quantum yields.

3.2.7 Isothermal Titration Calorimetry

Isothermal Titration Calorimetry (ITC) was carried out using a computer controlled Microcal VP ITC system (Microcal, USA). All samples used in the ITC experiments were performed in 10 mM HEPES, pH 7.9, 50 mM KCl, 5 mM DTT. The Gst-Tat protein and annealed duplex oligonucleotide (Table 1) were individually dialyzed extensively against the same buffer for three times. The buffer from last dialysis was used for washing the syringe and cell of instrument prior to experiment. The temperature of the cell was held constant for all experiments. Gst-Tat protein (10 μM) was placed in the cell (volume 1.436 mL) and the titrant NFκB DNA solution (40 μM, 300 μL) in the syringe. Precise injections with constant stirring of the sample were carried using a motor controlled plunger. Typically 30 injections of 10 μL each with equilibration time of 60 seconds were done. The calculation of kinetics and binding model was determined with best fit of data using the Microcal Origin software (version 5.0) provided by the manufacturer. (See Box 2)

3.3 Results

In earlier chapter it was shown that Tat can bind to NFκB enhancer DNA *in vitro* and *in vivo* and can modulate gene expression from NFκB enhancer containing promoter. In order to further characterize this specific interaction between Tat and NFκB enhancer DNA, biophysical studies were initiated, using recombinant native Tat, Tat fused to GST (GST-Tat) or GST fusion tag protein purified from *E. coli* BL21-DE3 as described in material and methods. Purity of protein was checked by SDS-PAGE, followed by silver staining and western blot.

Box 2
Isothermal titration calorimetry (ITC)

Calorimetry is a technique that utilizes direct measurement of the heat associated with a given binding. When measured at constant pressure it is equal to the change in enthalpy (ΔH) of that process. The calorimeters used to characterize the binding processes generally are based on constant temperature (isothermal) titration which measure the heat liberated during binding by dynamic power compensation. This means the energy liberated or absorbed is measured by the energy required to maintain a constant cell temperature. Dynamic power compensation improves the sensitivity and accuracy of operation. These types of instruments are known as isothermal titration calorimeter (ITC).

In ITC experiment, the macromolecule solution is located inside the sample cell and the ligand solutions in the injector syringe. A feedback control system supplies thermal power continuously to maintain the same temperature in both reference and sample cells. Any event taking place in the sample cell, usually accompanied by heat, will change the temperature in that cell and the feedback control system will modulate the power supplied in order to minimize such temperature imbalance. A sequence of injections is programmed and the ligand solution is injected periodically into the sample cell.

ITC is a unique technique that is capable of measuring simultaneously the association constant and the enthalpy of binding. Therefore a complete thermodynamic characterization of the binding process is possible. This can be obtained by using the basic thermodynamic relationships:

$$\Delta G = -RT \ln K_a = \Delta H - T\Delta S$$

Where ΔG is the Gibbs energy change, ΔS is the entropy change, R is the gas constant and T is the absolute temperature. K_a is association constant.

From a single experiment one can determine K_a, ΔH, and also the number of ligand binding sites on the receptor. Also, ITC provides information like the Gibbs energy which determines if a given binding process is thermodynamically favored or not and the strength of the interaction, along with its partition between enthalpy and entropy which is equally important.

ITC allows a further classification of ligands: according to their binding affinity and according to their enthalpy/entropy balance. It has been successfully shown that ITC can be used for characterizing very broad affinity systems (K_a from 10^{-2} to 10^{-12} M^{-1}). ITC is a method with exceptional capabilities of gaining very useful insights to thermodynamic information and characterizing binding processes.

Gel shift assay was used to reassess the DNA binding activity of purified recombinant Tat, GST and GST-Tat proteins prepared for biophysical studies. Only GST-Tat and native Tat protein specifically binds to NFκB consensus sequence, while five fold excess of control GST fusion tag protein failed to show any binding activity (Figure-2). This reconfirmed the specific binding activity of recombinant Tat proteins with NFκB enhancer DNA. To find changes in structural confirmation of Tat protein upon binding to DNA, circular dichroism spectroscopy (CD) was utilized. The binding interaction was further confirmed by recording circular dichroic (CD) spectra of Tat protein alone and after incubation with LTR NFκB wild type and mutated oligonucleotide. A negative band at 208 nm was observed, which on addition of wild type LTR oligonucleotide was reduced in intensity (Figure-3). But there was no major structural rearrangement in protein structure upon binding to DNA. This small but significant change is not observed with mutated oligonucleotide and is also not due to simple additive effect of the spectra of DNA and protein, since the computer addition spectra was different from the spectra of the complex. This definite change in signature supported specific interaction of NFκB enhancer sequence and Tat protein.

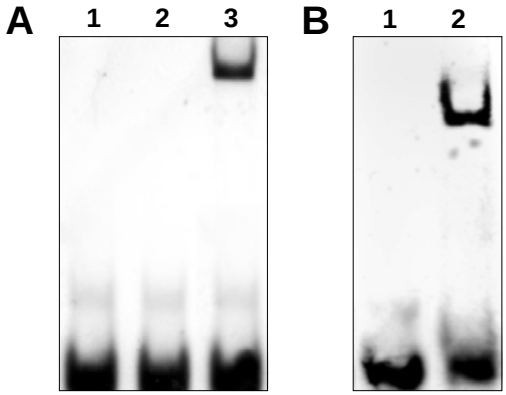


Figure 2: Gel shift assays for binding of purified GST-Tat and Tat protein with NFκB enhancer DNA sequences. (A) Lane1: Free probe; Lane 2: GST (15μg); Lane 3: GST-Tat (2.5μg); (B) Lane 1: Free probe; Lane 2: Tat (2.5μg).

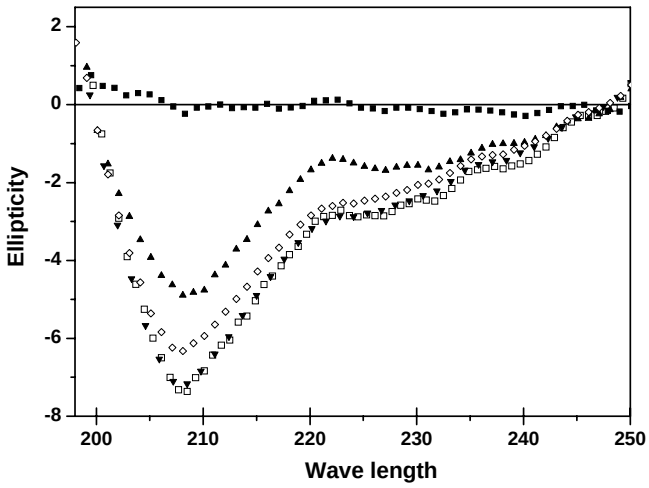


Figure 3: Circular dichroic spectra of Tat protein in presence of LTR NFκB oligonucleotide. A) CD spectrum of Tat protein, 30 μg/ml in PBS pH 7.4 (▼); B) change in CD signature of Tat protein in presence of LTR NFκB oligonucleotide, 0.1 nmole (▲); C) change in CD signature of Tat protein in presence of mutant LTR NFκB oligonucleotide, 0.1 nmole (◇); D) CD signature of LTR NFκB oligonucleotide, 0.1 nmole (■); e) computer addition spectra (□) of LTR NFκB oligonucleotide and Tat protein.

These proteins were then used for further biophysical studies by filter binding assay. It was observed that both Tat and GST-Tat could bind to NFκB DNA. The K_d for Tat: NFκB enhancer binding as obtained from filter binding assay was ≈3.05μM. No difference was obtained in K_d of interaction between GST-Tat and NFκB enhancer sequence ≈3.05μM (Figure-4). Since Tat is relatively unstable, with tendency to exhibit aggregation, all further studies were carried out using highly soluble and stable GST-Tat protein.

The stoichiometry of binding of GST-Tat to its DNA binding site was determined by fluorescence using fluorescein labeled synthetic oligonucleotides. Stock solutions of GST-Tat were titrated into DNA solution and monitored for change in fluorescence anisotropy. The anisotropy increased linearly with added protein in the initial part of the curve, suggesting that the initially added protein bound quantitatively to the DNA. However with increasing protein concentration, the anisotropy changes became smaller and finally

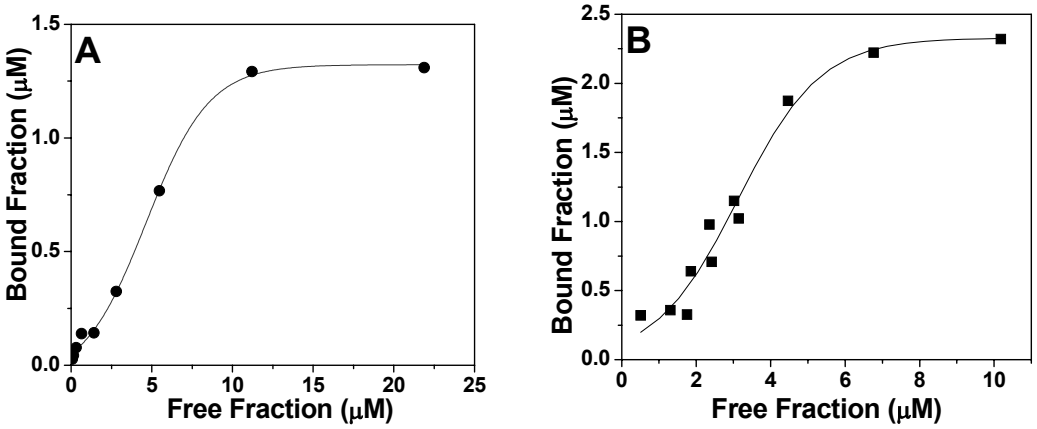


Figure 4: Determination of dissociation constant (K_d) using filter-binding assay. Varying concentration of radiolabelled oligo was incubated with Tat protein or GST-Tat protein. The complex was filtered through MF-Millipore Membrane Filter (HAWP01300). The amount of free and bound form of oligo was determined by radioactive scintillation counting. (A) Binding isotherm of GST-Tat (●); (B) Tat (■) protein shows similar affinity of binding.

reached a plateau indicating the complete saturation of the DNA fragment with GST-Tat. The intersection or the break point of the initial linear increase with the final anisotropy yielded the molar ratio of protein that was sufficient to saturate the DNA solution. The experimental data indicated that approximately two equivalents of GST-Tat protein was necessary to saturate the DNA solution (Figure-5). Thus GST-Tat protein is binding to DNA as a dimer. This is consistent with the earlier reports demonstrating that Tat peptides can form metal linked dimer (8-10). Gel shifts were individually carried out with single strand of NFκB enhancer DNA (sequence 3, Table 1) and its complementary sequence to rule out the binding of Tat to either of the single strands of DNA. No binding was observed with either of the single stranded DNA which confirmed binding of Tat dimer to duplex DNA (Figure-6).

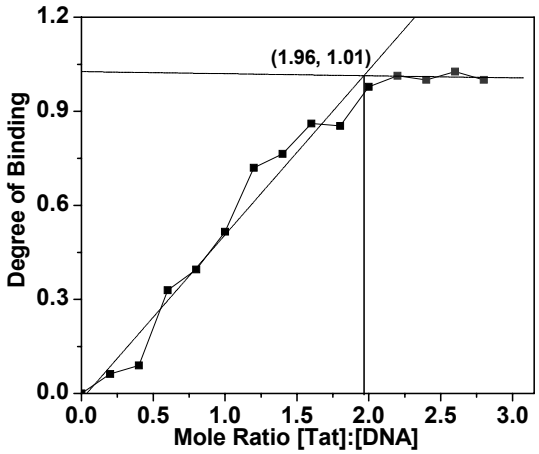


Figure 5: Stoichiometry of DNA: Tat interaction analyzed by fluorescence anisotropy. The anisotropy of DNA solution measured as a function of amount of protein added. Dotted lines are the linear regression of data.

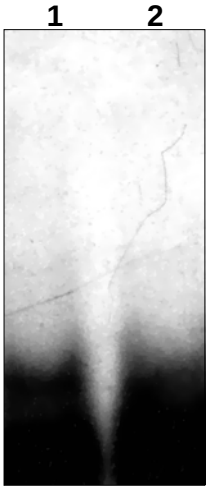


Figure 6: Gel shift assays for binding of purified GST-Tat protein with single stranded NFκB enhancer DNA sequences. Lane1: single strand of NFκB enhancer DNA sequence (sequence 3, Table 1) with GST-Tat (5μg); Lane2: complementary single stranded sequence DNA (reverse strand) with GST-Tat (5μg)

The binding strength was further characterized by fluorescence anisotropy measurements. No change in fluorescence intensity of oligonucleotide was observed upon complex formation of GST-Tat protein with DNA (Figure-7). However noticeable changes in fluorescence anisotropy were seen upon titration of GST-Tat protein to fluorescent DNA. The anisotropy increased with the concentration of proteins, reaching a constant value,

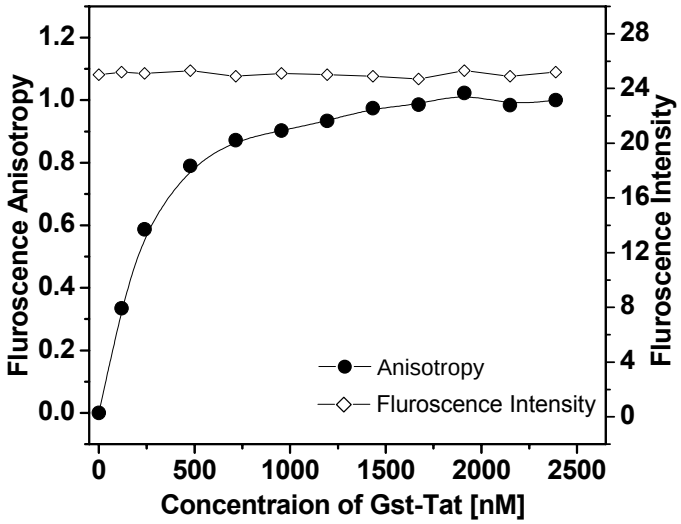


Figure 7: Fluorescence parameters for GST-Tat interaction with NFκB enhancer DNA sequence. The change in fluorescence anisotropy (●) and fluorescence intensity (◇) as function of increasing concentration of GST-Tat protein.

beyond some point. Since, the saturation of binding was observed beyond a stoichiometry of two equivalents of Tat to one equivalent of DNA, the data were fitted using 2:1 model for Tat: DNA interaction. The simple model can be determined by following equation

$$2 \text{ x Tat} + \text{DNA} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{Tat}_2\text{:DNA}$$

$$\text{Dissociation constant } K_d = \frac{k_2}{k_1} \qquad \text{Equation: 1}$$

The dissociation constant K_d was also determined at different salt concentrations by titration of fluorescein labeled wild type and mutant NFκB DNA and following changes in fluorescence anisotropy (Table 2).

Table 2 Effect of Salt concentrations on dissociation constant (K_d)

Serial No.	Concentration of KCl (mM)	K_d (nM) wild type	K_d (nM) mutant
1	25	89.5323 ± 0.5202	267.402 ± 0.506
2	50	265.355 ± 0.8442	2277.56 ± 1.015
3	75	435.027± 1.287	No binding
4	100	587.001 ± 1.495	No binding
5	125	1230.08 ± 1.443	No binding
6	150	1416.93 ± 1.905	No binding

At low salt concentrations (25 mM KCl) GST-Tat binds to wild type as well as mutant oligonucleotide and the binding affinity decreased with increase in salt concentration. The binding of GST-Tat to DNA is much weaker in case of mutant oligonucleotide. A three-fold decrease in binding affinity was observed at 25mM KCl with K_d 267.402 ± 0.506 nM and eight fold decrease in affinity with K_d 2277.56 ± 1.015 nM at 50mM KCL as compared with wild type oligo. As the salt concentration is further increased, Tat selectively bound only to wild type sequence while no binding is detected with mutant oligo. A significant increase in binding of wild type oligo was observed in presence of 1.25 mM $ZnCl_2$ with K_d 30.022 ± 1.08 nM. Control experiments were performed with GST and mutant Tat protein. Neither GST protein nor Tat C22G mutant protein display any binding to duplex oligo and there was no change in fluorescence anisotropy.

Anisotropy based competition assays were carried using unlabeled wild type and mutant NFκB oligonucleotides (Table 1) to determine the stability of protein-DNA complex (Figure-8). The anisotropy values were recorded for free oligo and that for 2:1 protein-fluorescent DNA complex, followed by addition of 100-fold excess of unlabelled

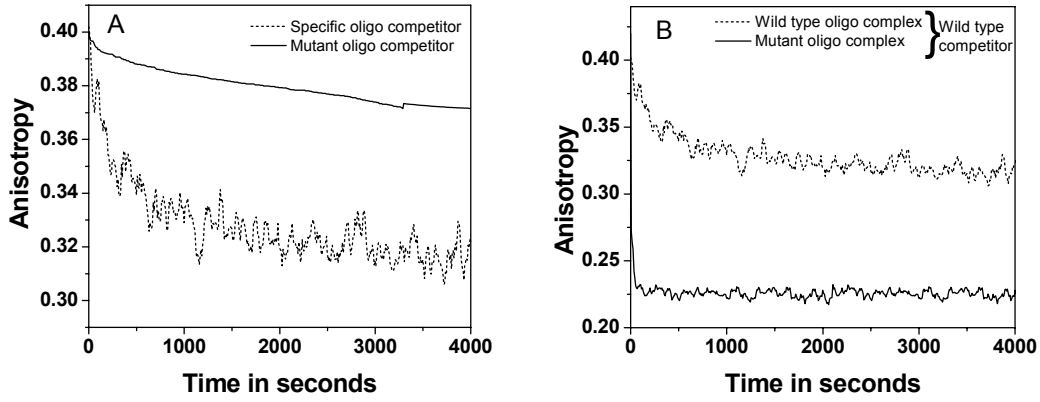


Figure 8: Kinetic specificity of GST-Tat: NFκB enhancer interaction using competition assay. The protein was incubated with fluorescein labeled oligo and allowed to form complex. 100-fold excess of unlabeled competitor oligonucleotide was added and change in anisotropy was recorded. A) Competition assay using unlabeled mutant (sequence 4) and specific oligo competitor (sequence 3), and wild type oligo (sequence 5) in complex. B) Competition assay using unlabeled specific oligo competitor (sequence 3) with complexes formed with either mutant oligo (sequence 6) or specific oligo (sequence 5)

competitor oligo to preformed protein-DNA complex. Wild type unlabelled oligo (sequence 3) effectively competes with the fluorescein labeled wild type oligonucleotide (sequence 5) to form the complex, while the mutant unlabelled oligonucleotide (sequence 4) failed to do so. In a reverse reaction, when mutant oligo (sequence 6) is used to form the complex, the wild type oligo (sequence 3) efficiently competes and displaces it from complex. This displacement reaction is very rapid in case of mutant oligo complex as followed by time course. The time required for decrease in anisotropy to basal level was much longer in the case of wild type oligo: protein complex. This demonstrates that Tat protein forms a more stable complex with the wild type DNA sequence than with mutant DNA.

These studies were complemented with the thermodynamic analysis by ITC (Figure-9) to analyse the mode of binding of GST-Tat protein to NFκB DNA. The heat changes were obtained by titration of the protein (receptor) solution with DNA solution (ligand, 10μL

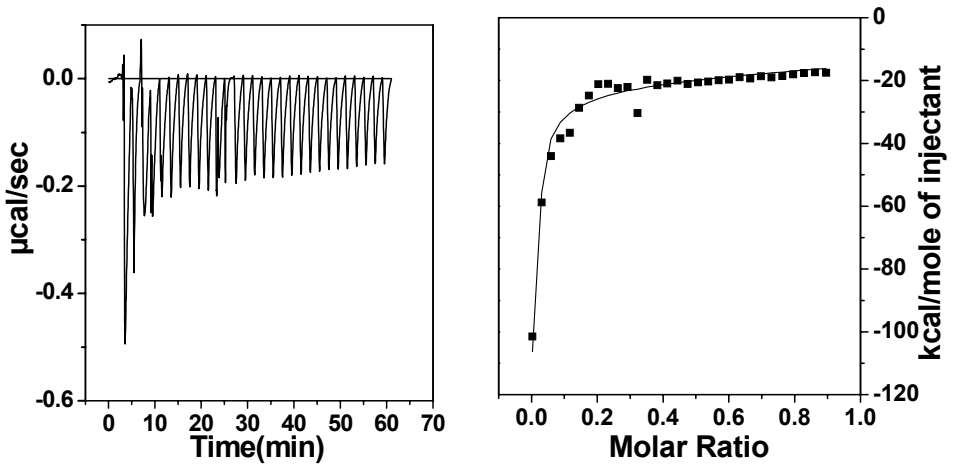


Figure 9: Thermodynamic analysis of GST-Tat interaction with NFκB DNA using Isothermal Titration Calorimetry. Isothermal titration calorimetry was done using GST-Tat and NFκB DNA as described. A typical data output after analysis is represented. The heat changes were obtained by titrating the protein (receptor) solution with DNA solution (ligand). The best fit of titration data was observed to be two sites sequential binding.

injection). The best fit of titration data was obtained for a two site sequential binding model (Figure-9, Table-3). This indicates a 2-step strong interaction of Tat protein to NFκB DNA.

Table 3: Thermodynamic Parameters obtained by ITC

Parameter	Value
K_1 (association)	1.066 x 10^5 M^{-1}
ΔH_1	-2.59 x10^5 Kcal/M
ΔS_1	-911.3 Kcal/°K/M
K_2 (association)	2.82 x10^7 M^{-1}
ΔH_2	2.262 x10^5 Kcal/M
ΔS_2	850.3 Kcal/°K/M

3.4 Discussion

HIV-1 transactivator protein, Tat is a unique transcription factor. Its primary function in viral life cycle is transcriptional activation of viral LTR promoter (11-15). It has an important role in transcriptional initiation and elongation (16). The transactivation function is primarily due to its interaction with the cognate target, TAR RNA. It interacts with TAR and recruits pTEFb complex to the viral promoter. Tat has no available crystal structure due to very unstable nature and several efforts in these directions have failed. The current understanding of the molecular mechanism of Tat: TAR interaction is based on techniques employing chemical modifications, cross-linking, affinity cleavage analysis and high-resolution foot printing of Tat: TAR complex. Based on the above analysis it has been proposed that amino terminus of Tat makes contact in the wide major groove of TAR RNA (2, 3). Due this TAR RNA conformation changes, which then wraps around the Tat peptide and induces rearrangement and ordering of complex structure. These studies suggest that neither the protein nor RNA has the correct structure before there interaction (3).

In earlier chapter, a new activity for Tat was demonstrated that it binds to NFκB enhancer DNA *in vitro* and *in vivo*. This binding leads to an increase in gene expression as evidenced by the reporter gene expression placed under the control of NFκB enhancer containing synthetic promoter. This observation adds another dimension to nucleic acid recognition properties of Tat. To gain further insight into mechanism of Tat: NFκB DNA interaction, biophysical studies were carried out using recombinant Tat. Studies with this protein was rendered difficult due to paucity of amount required for biophysical studies, compounded with the fact that Tat has a tendency to aggregate and precipitate at higher concentration and upon storage. It was observed that the precipitation of Tat protein is

enhanced in presence of DNA. Hence a more soluble GST-Tat fusion protein was utilized for these studies. Since GST alone does not bind to NFκB DNA as seen by gel shift assay and filter binding assay, the observed biophysical changes are consequence of Tat-DNA specific interactions.

The binding of Tat protein to target NFκB enhancer DNA oligonucleotides was followed by fluorescence anisotropy measurements. A substantial increase of low anisotropy value in the free oligonucleotide upon addition of Tat protein is indicative of specific protein-DNA interaction. This arises from freezing of the mobile fluorophore in DNA on interaction with the protein. Two equivalents of GST-Tat protein molecule were necessary for complete complexation with the target DNA. In earlier chapter it was demonstrated that the protein binding to DNA is increased in presence of Zn^{2+} ion and a transactivation negative mutant of Tat (cysteine 22 glycine) fails to bind the NFκB enhancer DNA (15). The cysteine residues have been demonstrated to be critical for dimerization of protein. Metal ion linked dimerization of Tat protein has been observed in literature (8, 9). These results overall suggest that Tat interacts with DNA as a dimer. The effect of ionic strength on dissociation of Tat: NFκB DNA complex was studied at different salt concentrations. Increase in KCl concentration to physiological range lead to specific binding of Tat, indicating that Tat-DNA interaction is feasible in physiological scenario.

With the above data, one cannot distinguish between possibilities of bindings of two monomers independently or in a single binding step of a preformed dimer. To delineate the problem of simple or cooperative binding, isothermal titration calorimetry was utilized. The protein solution was titrated with the DNA ligand, which leads to significant changes in entropy and enthalpy caused by binding. The best fit of data was obtained for a 2:1 protein:

DNA binding and the derived enthalpic and entropic values suggests that the binding of Tat to be a 2 step process, with the initial phase being an exothermic process while second binding phase an endothermic process. But this interpretation is to be treated cautiously since Tat protein aggregates in presence of DNA and ITC experiments do involve prolonged incubation of complex till saturation is achieved. Given the instability of protein and the tendency of complex to aggregate upon prolong incubation; the simple treatment of data from titration or saturation binding is inadequate to decipher the exact mechanism of binding.

Multifunctionality in macromolecular association and functions is an emerging feature of many proteins that bind to nucleic acids. The existence of transcription factors that bind to both DNA and RNA provides an interesting puzzle (17). Recent data on some DNA binding transcription factors indicate dual nucleic acid binding specificities, though little is known about the relation of alternative binding to biological roles. A classic example is the well-studied p53, which has two nucleic acids binding domain with different specificity (18), with evidence of p53 binding to RNA and subsequent regulation of translation of mRNA transcripts for few genes. I have earlier shown that Tat has structural homology to p53 family of transcription factors, and possesses structural motifs homologous to the N-terminal domain of the mouse and human NFκB p50 subunit. It is interesting to note here that even p65 subunit of NFκB protein has been shown to have RNA binding property (19, 20). I have previously shown the direct and specific interaction of Tat with isolated and chromatinzed NFκB enhancer elements of viral promoter. While Tat is one of the few transcription factors whose primary target is an RNA molecule, the specific DNA binding gives additional leverage to act as a master regulator, which orchestrates not only the steps

of viral life cycle, but also seems to take control of host transcription machinery (21, 15). While the relation of Tat binding to DNA and p53 binding to RNA is obscure, the underlying principle could be a universal rule for context dependent functioning of transcription factors.

Tat plays a critical role in recruiting HATs and chromatin remodeling of integrated LTR, which is essential for gene expression and reactivation from latency (22-27). In this context, a direct interaction of Tat with enhancer elements in promoter could play an important role in increased transcription initiation. Evidence provided in this chapter shows that Tat binding to DNA is specific, strong and occurs in submicromolar concentration. This level of expression has been observed in the infected cells (28). Tat binding specifically to NFκB enhancer sequence supports its plausible role in TAR independent transactivation and modulation of cellular gene regulation.

3.5 References

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Chapter4: Role of Tat in modulation of transcription in HIV-1 infected cells

4.1 Introduction

Efficient packaging of DNA into the cell is one of the most remarkable characteristics of all eukaryotic organisms. The human genome, which is about $3x10^9$ base pairs of DNA and a total length of about 2 m, is packaged into a nucleus as little as 6-8 μm in diameter. This packaging is achieved by compaction of DNA into highly condensed structure, referred to as chromatin. Chromatin allows the essential processes of DNA replication, repair, and transcription to proceed while maintaining a considerable degree of compaction.

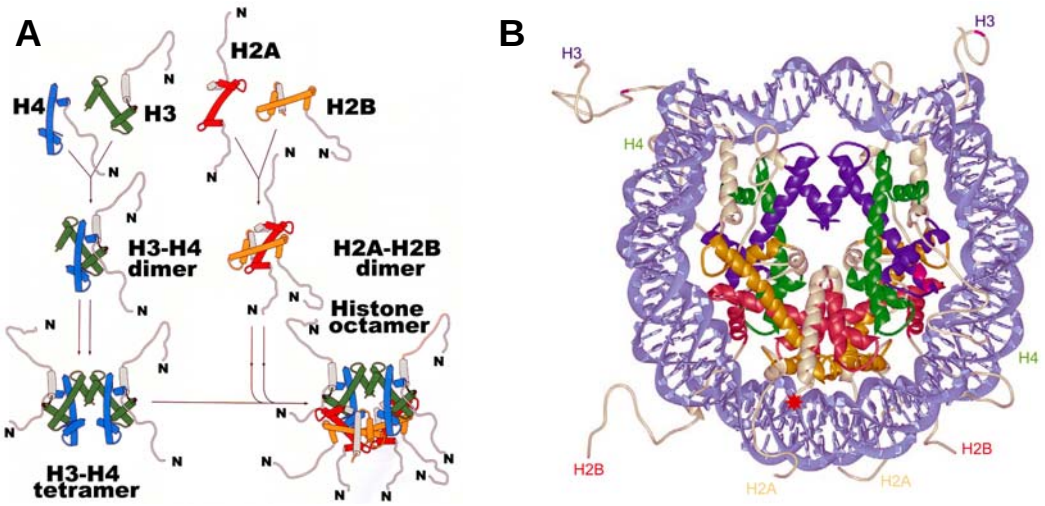

Figure 1: The assembly of a histone octamer and overall structure of the nucleosome core particle. A) The histone H3-H4 dimer and the H2A-H2B dimer are formed by the mechanism called handshake interaction. An H3-H4 tetramer forms the scaffold of the octamer onto which two H2A-H2B dimers are added, to complete the assembly. All eight N-terminal tails of the histones protrude from the disc-shaped core structure. The histone tails are unstructured and are highly flexible. (Adapted from Alberts, B. *et al*. (2002) "Molecular biology of the cell", 4th edition, publisher Garland Science, USA.) B) The nucleosome core particle viewed down the super helical axis. The histone fold domains of H2A, H2B, H3, and H4 are colored respectively and DNA is shown in light blue. H3 residues that are responsible for selective replication-coupled assembly are shown in magenta. The red star indicates the site of ubiquitination in yeast. (Adapted from Luger, K., (2003) Structure and dynamic behavior of nucleosomes. *Curr. Opin. Genet. Dev.* 13:127–135)

Nucleosomes are the building blocks of chromatin and are object of active research in scientific community. Nucleosome is a very dynamic structure consisting octamer of constituent histone proteins Histone 3, Histone 4, Histone 2A, and Histone 2B with DNA wrapped around it (Figure-1A).

Chromatin structure is modulated by the covalent modifications of the core histones in nucleosomes due to the action of chromatin remodeling complexes. The amino terminus of histones protrudes out from the core nucleosome (Figure-1B), where they can be modified post-translationally by acetylation, phosphorylation, and methylation, each modification differently affecting their charge and function (Figure-2).

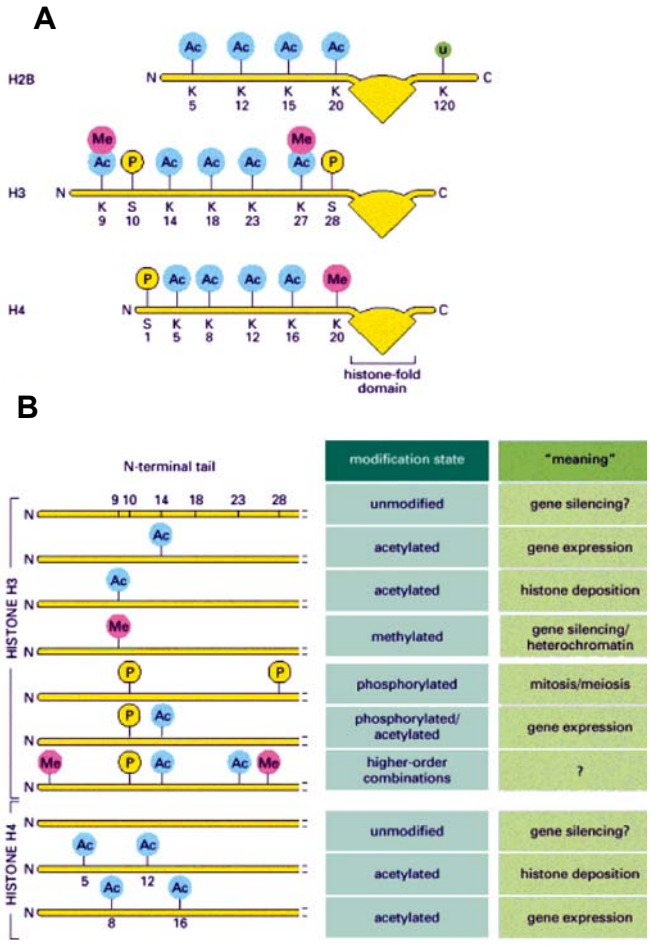

Figure-2: Covalent modification of core histone tails and the histone code hypothesis. A) Known modifications of the four histone core proteins are indicated: Me = methyl group, Ac = acetyl group, P = phosphate, u = ubiquitin. **B**) A histone code hypothesis. Histone tails can be marked by different combinations of modifications. According to histone code hypothesis, each marking conveys a specific meaning to the stretch of chromatin on which it occurs. Only a few of the meanings of the modifications are known. Modification of a particular position in a histone tail can take on different meanings depending on other features of the local chromatin structure. Some histone tail modifications are interdependent. (Adapted from Alberts, B. *et al*. (2002) "Molecular biology of the cell", 4th edition, publisher Garland Science, USA.)

In particular, histone acetylation at the promoter of genes, mediated by histone acetyltransferases (HAT), has been shown to be necessary, albeit not sufficient, for transcriptional activation (1-3). Upon stimulation HATs are recruited to promoter by various activator proteins (transcription factors) which then acetylate histone protein. This results in decondensation of the chromatin structure. Opening of chromatin leads to recruitment of transcription machinery and expression of genes. The recruitment of transcription machinery is aided by mediator proteins. Similarly the repressor proteins

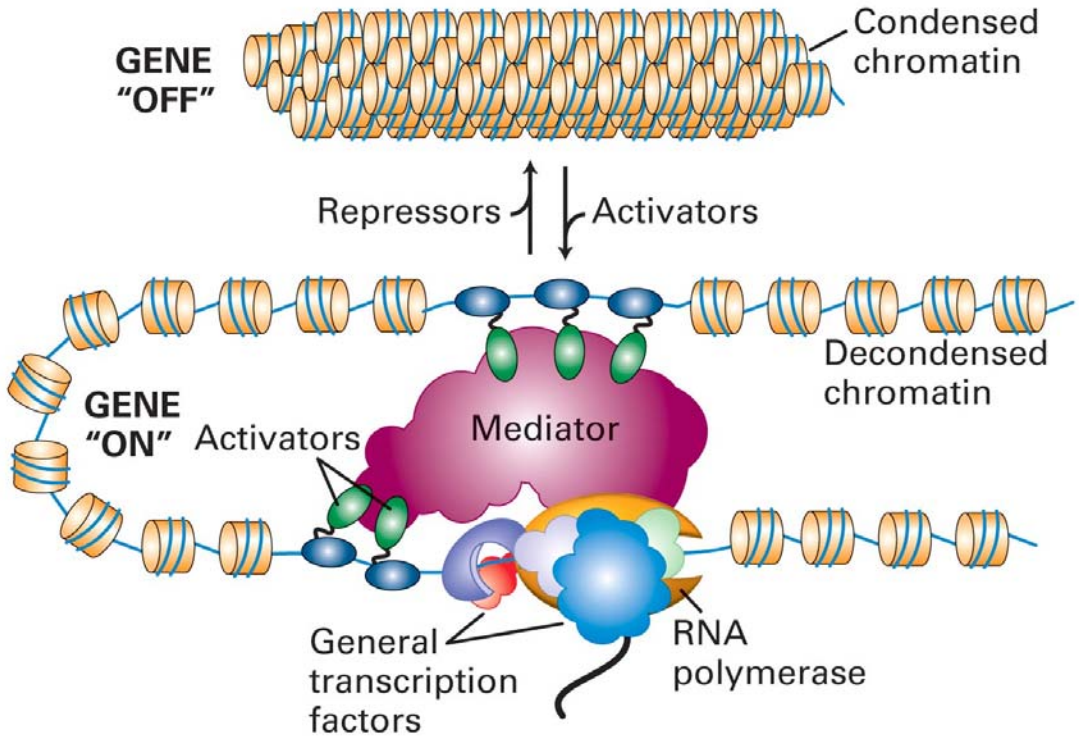

Figure 3: Model of gene activation based on histone modification. Activator proteins recruit HATs and other components of chromatin remodeling complex to the condensed chromatin. These modify the N-terminal tail of histones in the nucleosomes which results in opening of the chromatin structure. Subsequently expression of the gene occurs due to recruitment of transcription machinery to the promoter. Conversely, repressors recruit HDACs to active gene promoters which modify the histone tails and causes condensation of chromatin. Due to condensation the access of transcription machinery to the promoter is restricted which results in repression of expression.

recruit HDACs to the promoter which deacetylate the histone and result in tight wrapping of DNA in nucleosome particle. This leads to repression of gene expression (Figure-3).

Gene regulation is a complex process controlled by many factors. These factors are assembled into multiprotein complexes, contributing to specific gene regulation events. Regulatory viral and cellular proteins modulate LTR mediated expression of HIV-1 proviral genome. In cells infected with HIV-1, proviral genome is tightly packaged into chromatin. Nuclease-accessibility studies *in vivo* (4-6) and *in vitro* using the HIV promoter reconstituted into chromatin (7, 8) have shown that the 5' LTR is always precisely organized into two distinct nucleosomes (Figure-4), termed nuc-0 and nuc-1 (9-11). Chromatinzed provirus in the 5' region of the HIV-1 genome is found to be sensitive to DNase I digestion. Two regions, called hypersensitive sites 2 and 3 (HS 2, 3), are associated with the promoter in the U3 region (HS2 encompassing nt 223-325 and HS3 nt 390-449).

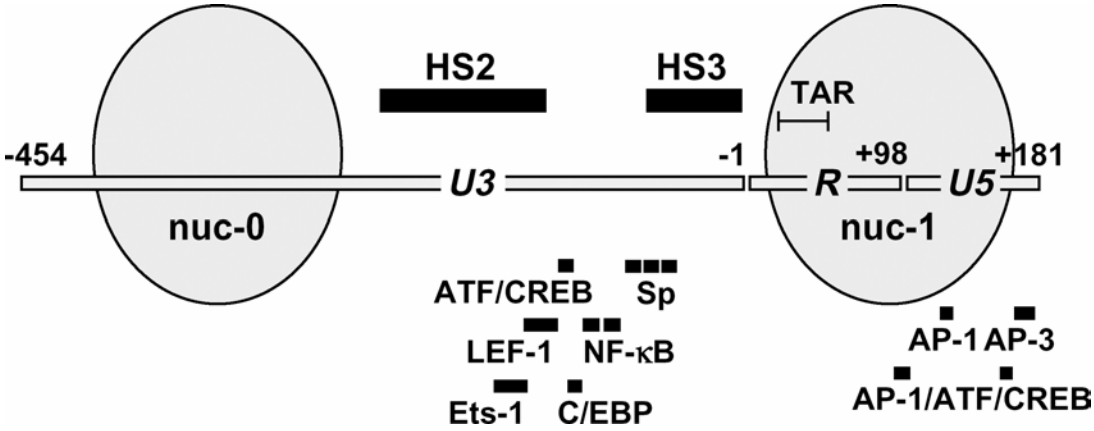

Figure 4: LTR is organized into nucleosomes *in vivo*. Positions of the DNase I hypersensitive regions (HS2 and HS3) and locations of the nucleosomes (nuc-0 and nuc-1) along the HIV-1 LTR are depicted. The locations for the binding sites for transcription factors implicated in chromatin-dependent viral gene expression are also shown. (Source: Krebs, F. C. *et al*., (2001) in *HIV Sequence Compendium 2001,* pp. 29-70.)

Emerging data now suggests that dynamic regulation of chromatin at the LTR adds an additional level of complexity to the regulation of HIV expression. The viral long terminal repeat (LTR), which acts as a very strong promoter when analyzed as naked DNA *in vitro* (12), is almost silent when integrated into the cellular genome in the absence of stimulation (11, 12). During transactivation, nuc-1 located at the site of transcription initiation, is specifically and rapidly unwound indicating its importance in the maintenance of a transcriptionally silent promoter. The proximity of nuc-1 to the transcription start site and its displacement during transactivation suggest that chromatin plays a crucial role in the suppression of HIV-1 transcription during latency (4, 5). Role for nucleosome organization in regulation is further supported by the observation that LTR activity is increased upon treatment with specific HDAC inhibitors such as sodium butyrate and trichostatin-A (13-21).

Inhibition of HDACs is sufficient for transactivation of the HIV-1 LTR, implying that nucleosomes at the HIV-1 LTR are constitutively deacetylated. Several studies have shown that HDACs may be targeted to the HIV-1 promoter by specific transcription activators. Several E-box binding proteins have been shown to interact with HDAC-1 and recruit it to LTR (22-25). Likewise, the Mad-Max heterodimers, which are bHLH-Zip proteins, have been shown to repress transcription via recruitment of the mSin3-HDAC co-repressor complex (26-27). YY1 and LSF transcription factors also cooperate in the repression of the HIV-1 LTR and viral production via recruitment of HDAC1 to nuc-1 (28-31). Also recent reports demonstrate that SP1 and p50 can interact with HDAC-1 indicating their role in repression (32-38). This again indicates a possible mode for recruitment of HDAC-1 to LTR. Jordan and co-workers in an elegant study have shown that HIV reproducibly

establishes a latent infection after acute infection of T cells *in vitro* (39). Recent findings have emphasized that the nonproductive nature of the infection in resting $CD4^{+ve}$ T cells with integrated HIV-1 DNA is not the result of the nature of the integration site (40). This could be then a sum total of many factors including chromatin remodeling at LTR in which HDAC-1 may act as the ultimate switch of repression (41). Lusic and co-workers recently demonstrated the presence of HDAC-1 at LTR promoter in latently infected U1 monocytic cell line (42). But in another study, Thierry and co workers could not detect role of HDAC-1 in reactivation of HIV-1 in latently infected ACH2, U1 and OM-10.1 cell lines (43). However both these studies in infected cells focused mainly on reactivation of latency and present contradicting role for HDAC-1. These studies demonstrate a definite role for Tat in recruitment of HATs and acetylation of histones which results in chromatin remodeling at LTR. Albeit no studies have been carried out which demonstrate chromatin regulation at LTR in acute infection.

Promoter occupancy by transcriptional activators is a hallmark of active transcription. Both LTR promoter activity and the function of viral regulatory proteins require the activity of cellular components, thus the virus remains highly dependent on the metabolic state of the host cell. A better understanding of the role of chromatin in the regulation of HIV expression may lead to new therapeutic strategies against proviral genomes that are being actively transcribed or those which are quiescent. Cellular factors that bind to the nucleosome free region upstream of nuc-1 are thought to regulate the remodeling of nuc-1 and initiation of transcription (44). HIV-1 genome encodes three viral products Nef, Vpr, and Tat, which are known to regulate the transcription. The activity of these factors is

largely dependent on cellular co-activators, which may also regulate HIV-1 chromatin architecture (44).

Tat is known to recruit histone acetyl transferases (HATs) such as p300 and p300/CBP associated protein P/CAF to the chromatin, which results in histone acetylation and opening of chromatin (42, 44-49). Tat derepresses the integrated HIV-1 chromatin structure, aiding in activation of transcription from transcriptionally silent proviral promoter. In integrated promoter both NFκB and SP1 sites are critical for Tat mediated transactivation and chromatin remodeling. Deletion mutants of these enhancer sequences are shown to be unresponsive to Tat transactivation (6, 48).

In earlier chapter, I have shown convincing evidence for the presence of Tat on integrated LTR in infected cells. Based on this data, further studies were initiated to look at the role of Tat in both viral and cellular expression at different time points post infection. This chapter describes studies on histone modification mediated chromatin modulation at proviral promoter during the course of viral infection and its correlation to level of Tat recruitment to the promoter. The histone modifications are characterized by quantitative chromatin immuno-precipitation assay (qChIP) using real time polymerase chain reaction. Similar studies have been also carried out with IL-2 gene promoter, which is known to be modulated by Tat in infected cells (49-54).

4.2 Materials and Methods

4.2.1 Antibodies, Plasmids, Oligonucleotides

All antibodies required for ChIP assays were purchased from commercial sources. Antibodies to Acetyl-Lysine, and RNA Pol II were obtained from Santacruz, USA.

Antibodies to Histone-3, Acetylated-Histone-3, Histone-4, Acetylated-Histone-4, and Histone deacetylase-1 was obtained from Cell Signaling, USA.

Plasmids encoding luciferase reporter gene under the control of Interleukin-8 promoter (1.5 Kb fragment, pIL8-Luc), was a kind gift from Prof. Marie A. Buendia (55). pEGFP-N1 was obtained from Clontech, USA. pCDNA-Tat expressing wild type Tat protein has been described previously.

All oligonucleotides for PCR were purchased from commercial supplier (Genomechanix, USA). Oligonucleotides were purified to more than 95% purity on a C18 reverse phase HPLC column using triethyl ammonium acetate buffer.

Table 1: List of oligonucleotide sequences used in this study

Name	Sequence	Region
Enhancer F	**5' CCTGCATGGAATGGATGACC**	HIV-1 enhancer
Enhancer R	**5' CGCCCAGGCACGCTCC**	HIV-1 enhancer
Nuc1 F	**5' TGTACTGGGTCTCTCTGGTTAG**	HIV-1 nuc-1
Nuc1 R	**5' ACCAGAGTCACACAACAGACG**	HIV-1 nuc-1
TIMM 13 F	**5' GACTGGAAACTTTTTTGTAC**	B2 lamin region
TIMM 13 R	**5' TAGCTACACTAGCCAGTGACCTTTTTCC**	B2 lamin region
IL-2 F	**5' GGGCTAATGTAACAAAGAGGGATT**	Interleukin-2 Nuc
IL-2 R	**5' AACCCATTTTTCCTCTTCTGATGA**	Interleukin-2 Nuc
IL-8 F	**5' GCATACAATTGATAATTCACC**	Interleukin-8 gene
IL-8 R	**5' CTTGTGTGCTCTGCTGTCTC**	Interleukin-8 gene

4.2.2 Transient transfection and Reporter assay

All transfection experiments were done as described in section 2.2.8.1 and reporter assays were performed as described in section 2.2.9

4.2.3 HIV-1 infection

2 x 10^7 CEM-GFP cells (56) were infected with 0.5 MOI of HIV-1 NL4.3 virus (57) along with 1μg/ml polybrene (Sigma, USA) for 4 hours at 37°C in a humidified incubator with 5% CO_2. These cells were then transferred to fresh RPMI 1640 with 10% FCS and incubated at 37°C. The infected cells were maintained for a period of 32 days. The CEM-GFP cell line has stably integrated Green Fluorescent Protein (GFP) gene under the control of HIV-1 LTR promoter, expression of which is induced by HIV-1 infection due to Tat mediated transactivation. The progress of infection was monitored visually by fluorescence microscopy and quantitation of GFP fluorescence in Fluoroskan *Ascent* FL microplate reader (Labsystems, Finland) and followed by p24 assay of the culture supernatant using p24 antigen ELISA kit (Perkin Elmer life science, USA).

4.2.4 PCR

Purified DNA samples were used as template for amplification. The thermo-stable enzyme *Taq* DNA polymerase was purchased from Invitrogen, USA and Advantaq was purchased from Clontech, USA. The PCR reactions were carried out using buffers provided by the manufacturer in Master cycler gradient thermal cycler (Eppendorf, Germany).

4.2.5 Quantitative chromatin Immunoprecipitation (qChIP) Assay

The ChIP assay was performed as described in section 2.2.10. The amount of DNA in the chromatin immunoprecipitate was estimated by quantitative PCR. Essentially all the PCR detections were carried out in iCycler Real time PCR detection system (Biorad, USA).

The DNA was isolated and resuspended into equal volume (20 μL) of autoclaved deionized water.

4.2.5.1 Real Time PCR

All quantitative PCR reactions were carried out in iCycler Real time PCR instrument (Biorad, USA) using Advantaq (Clontech, USA). 0.2 X SYBR Green I (Molecular Probes, USA) was used to monitor the progress of PCR amplification reaction. Amplification protocols were optimized in PCR buffer containing 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl, 2 mM $MgSO_4$, 0.1% Triton X-100, pH 8.8 at 25°C (NEB, USA) using various combinations of $MgCl_2$ concentration and cycling conditions. All the PCR reactions were spiked with 5 nM Fluorescein dye used as internal reference control for normalization of fluorescence.

4.2.5.2 Estimation of immunoprecipitate

Serial dilution of purified PCR product was used as template in real time PCR. The relative proportions of template were determined based on the threshold cycle (C_T) value for each PCR reaction. C_T value is defined as the cycle at which fluorescence raises to 10 times above the mean standard deviation of background levels in all reaction wells. C_T values for each reaction were plotted against the log values of the starting template amount to generate standard curve. The quantity of immunoprecipitated DNA was calculated using the C_T value obtained in real time PCR and the standard curve generated for the respective primer pair.

4.3 Results

Retroviral life cycle involves integration of genome into host DNA. Following integration into the host genome, the proviral DNA is organized into chromatin. Due to this integration, HIV-1 LTR is organized into nucleosomes and functions like a host promoter. This provides an excellent opportunity for deciphering regulatory networks involved in the control of HIV-1 LTR in chromatinzed environment as well as some understanding of regulation of cellular promoters. With this view studies were performed with HIV-1 infected T cell line CEM-GFP using quantitative ChIP assay.

CEM-GFP cell line has integrated LTR promoter, regulating the expression of GFP (41). There is very low expression of GFP in absence of infection which increases to very high levels upon infection due to Tat mediated transactivation. The progress of viral

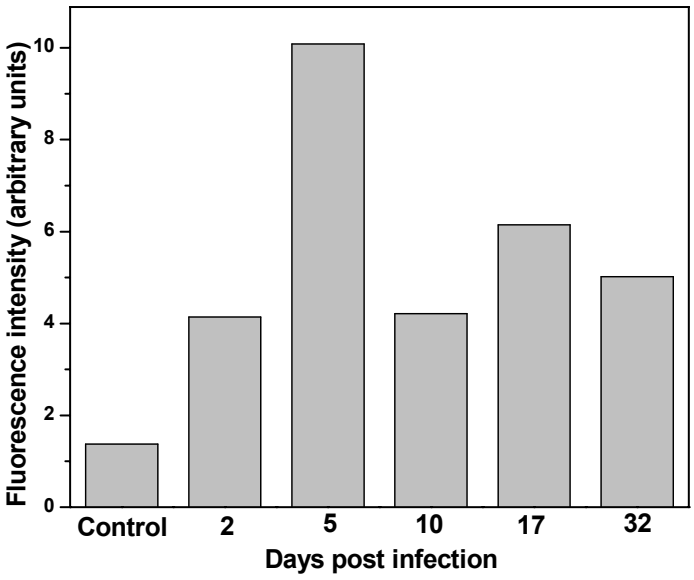

Figure 5: Level of GFP expression during the course of HIV-1 infection in CEM-GFP cells. HIV-1 infected CEM-GFP cells were harvested and expression of GFP was determined by quantification of the fluorescence intensity of the cell lysate. Equal amount of protein was used for quantitation of GFP fluorescence. Data shown is mean of two independent experiments

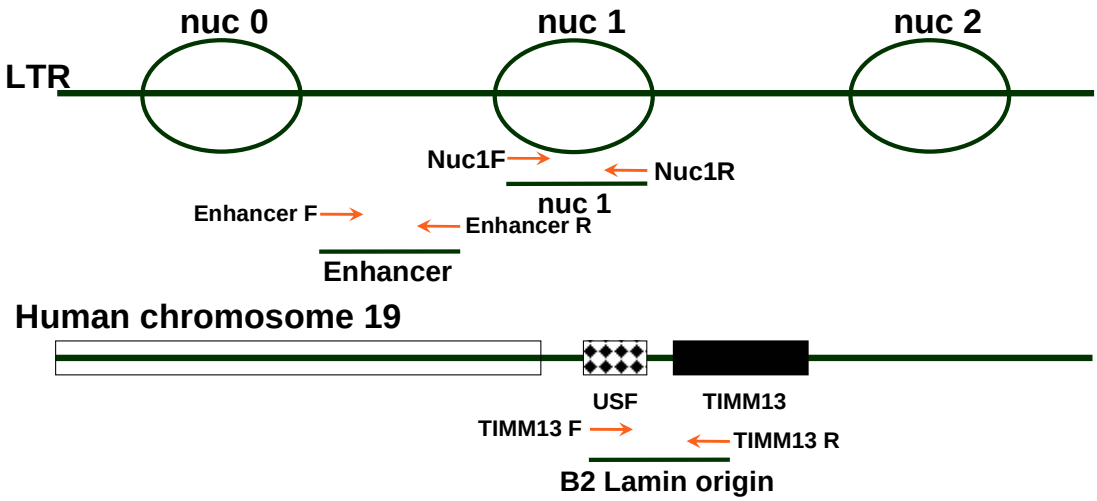

Figure 6: Schematic structure of the integrated HIV-1 LTR promoter and the control genomic region on human chromosome 19. On the promoter sequence the position of nucleosomes and regulatory region and the primer pairs used for PCR amplification are indicated by arrows.

infection can be monitored visually and also quantified by fluorescence spectroscopy. This gives a good estimate about level of infection achieved and hence also an estimate of transcription from viral promoter. Thus, this model provides an excellent opportunity to study the LTR promoter of virus in absences of Tat or the virus. In acute infection this model system provides a platform for the study of Tat mediated chromatin modulation at the LTR and cellular promoters which are regulated by Tat. The cells were infected at 0.5 MOI of the HIV-1 NL4.3 virus so as to obtain high infection efficiency in a shorter time point. The level of infection in the cells was then monitored visually by fluorescence microscopy. All the time points for this study were selected based on visual monitoring of infection. There is an initial increase in GFP expression, which latter subsides with further progression of infection (Figure-5). At various time points post infection cells were harvested and cross-linked with formaldehyde and chromatin was prepared for ChIP analysis.

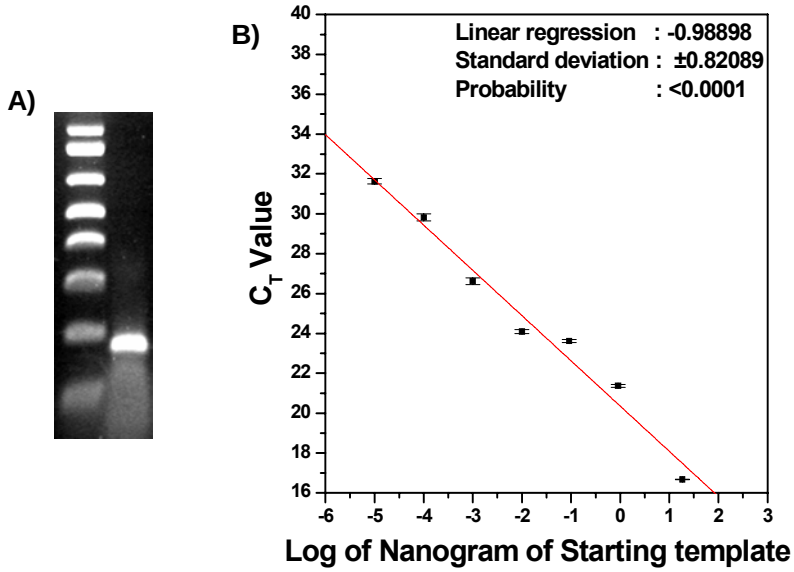

Figure 7: Real time PCR with primers flanking Tat binding site in HIV-1 LTR. A) Primers Enhancer F and Enhancer R were used to amplify the NFκB region in the LTR. A specific band for amplified product is obtained. **B)** Gel extracted band was used as template to generate the standard curve by real time PCR.

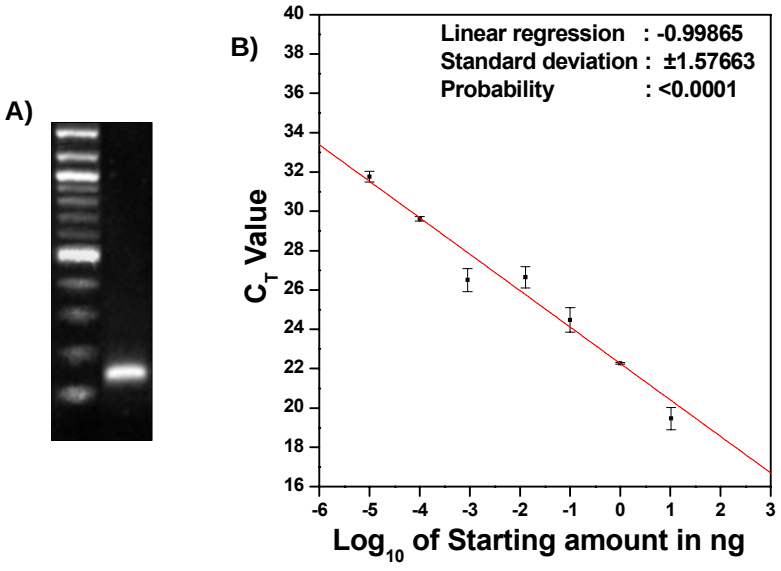

Figure 8: Real time PCR with primers flanking promoter region of mitochondrial inner membrane translocase 13 (TIMM13) gene. A) Primers TIMM13F and TIMM13R were used to amplify the promoter region of the TIMM13 gene. **A**) Specific band for amplified product is obtained. **B)** Gel extracted band was used as template to generate the standard curve by real time PCR.

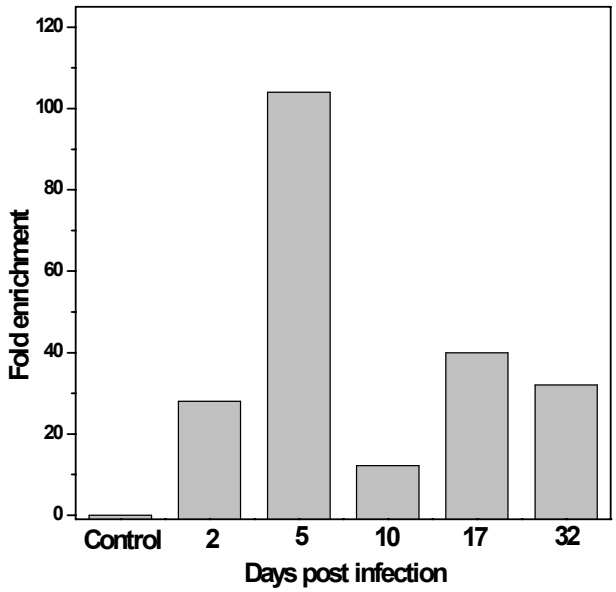

Figure 9: Tat occupancy at HIV-1 LTR during the course of infection. The fold enrichment was calculated by normalizing the amount of LTR DNA immunoprecipitated by Tat antibody to amount of TIMM13 DNA in the same sample. Data shown is mean of two independent experiments.

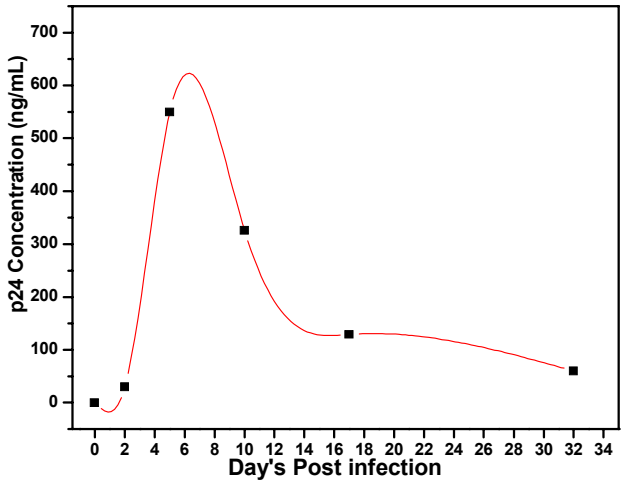

Figure 10: Level of HIV-1 gene expression during course of infection. The viral production was determined by amount p24 antigens present in culture supernatant. Data shown is mean of two independent experiments.

4.3.1 Chromatin modulation at LTR

The quantitative level of Tat present at integrated LTR was determined using real time PCR. Primers specific for lamin B2 origin sequence encompassing the promoter region of mitochondrial inner membrane translocase-13 (TIMM13) gene which, as reported in previous studies, is not modulated during HIV-1 infection (42) was used as internal control (Figure-6). These same primers were used as internal control for normalization in all further ChIP experiment in the current study. Initially optimization of amplification conditions was carried out for real time PCR for various primer combinations to obtain a specific product and generate linear standard curves.

The primers flanking the Tat binding site were used to find the levels of Tat occupancy in LTR region at various time points during the course of infection. Robust amplification was obtained in PCR (35 cycles: 94°C for 30 sec, 55°C for 60 sec, 72°C for 60 sec) using primers Enhancer F and Enhancer R specific for Tat binding region in presence of 4 mM $MgCl_2$. An internal control PCR (35 cycles: 94°C for 30 sec, 55°C for 60 sec, 72°C for 60 sec) for promoter of gene TIMM13 was used for normalization in ChIP assay. (Figures 7 and 8)

The levels of Tat bound to LTR chromatin was estimated by qChIP assay. The chromatin preparations were immunoprecipitated using Tat specific antibody. The amount of LTR DNA in the precipitated samples was estimated using C_T values from real time PCR. Time dependent variation in Tat occupancy was observed. (Figure-9) There is an initial increase followed by subsequent decrease in level of Tat binding to LTR. The fold enrichment of LTR specific ChIP was calculated by dividing the amount of experimental promoter DNA bound by Tat by control TIMM13 DNA bound by Tat in particular

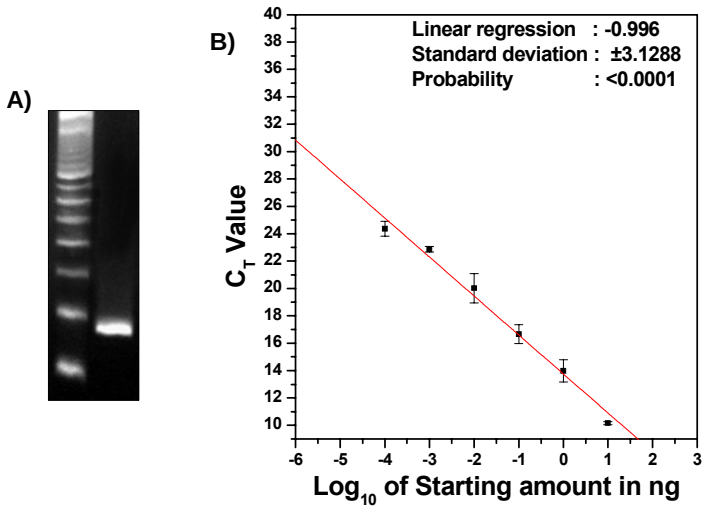

Figure 11: Real time PCR with primers flanking nuc-1 region of the HIV-1 LTR. **A)** Primers Nuc1F and Nuc1R were used to amplify the nuc1 region in the LTR. A specific band for amplified product is obtained. **B)** Gel extracted band was used as template to generate the standard curve by real time PCR.

precipitate. To obtain quantitative information on effect of Tat on level of gene expression p24 antigen ELISA was utilized (Figure-10). An excellent correlation of HIV gene expression and viral production was observed with Tat occupancy of the promoter. This correlation also extends to GFP expression data shown earlier (Figure-5).

Tat is known to displace HDACs and recruit HATs and modulate chromatin at viral LTR, which leads to increase in gene expression. Role of HDAC-1 in modulation of LTR has been initially characterized by various research groups (reviewed in 44). To study this in current model by qChIP, real time PCR conditions for amplification were optimized using primers flanking the nuc-1 position to obtain a specific product and generate a linear standard curve. Robust amplification was obtained with PCR (35 cycles: 94°C for 30 sec, 55°C for 60 sec, 72°C for 60 sec) using primers Nuc1 F and Nuc1 R specific for nuc-1 region in presence of 4 mM $MgCl_2$ (Figure-11) A time dependent change in HDAC-1

occupancy of LTR was observed (Figure-12). HDAC-1 recruitment has been directly implicated to histone acetylation, and opening of chromatin (41).

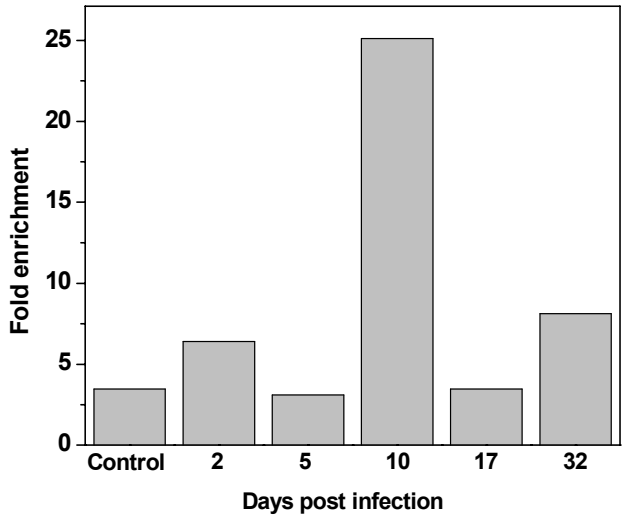

Figure 12: HDAC-1 occupancy at HIV-1 LTR at different days post infection. The fold enrichment was calculated by normalizing the amount of LTR DNA immunoprecipitated by HDAC-1 antibody to amount of TIMM13 DNA in the same sample. Data shown is mean of two independent experiments.

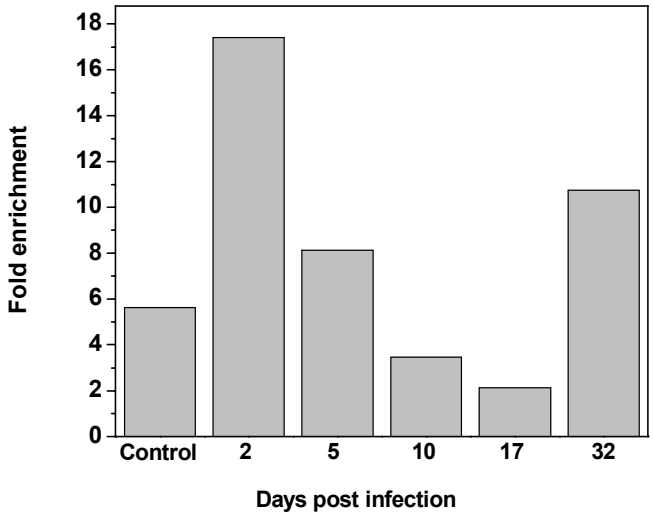

Figure 13: Level of acetylation at HIV-1 LTR during course of HIV-1 infection. The fold enrichment was calculated by normalizing the amount of LTR DNA immunoprecipitated by acetylated-lysine specific antibody to amount of TIMM13 DNA in the same sample. Data shown is mean of two independent experiments.

A good correlation of HDAC-1 recruitment with of CEM-GFP fluorescence and p24 expression was observed in infected cells. Uninfected cells have minimum basal levels of HDAC-1 at LTR, this level fall with progress of infection to minimum on Day 5 where maximum expression of GFP is observed. As infection further progresses to Day 10 there is decrease in fluorescence with concomitant increase in recruitment of HDAC-1. Beyond Day 10, a minimal level fluorescence which is slightly higher than basal level of uninfected cells is observed.

Histone acetylation has always been associated with increased transcriptional activity of the promoter. This increase in transcription is due to relative ease of access for transcription machinery to the open DNA of promoter. Upon acetylation, the DNA wrapped around the nucleosome is unwound which is a signal for assembly of transcription complex. A variation in HDAC-1 level in conjunction with GFP expression was observed. Hence, experiments were carried out to study the effect of this variation on global acetylation status of LTR nuc-1 using pan–acetyl antibody specific for acetyl modification of lysine residues of histones. A HDAC-1 level dependent variation in acetylation status of nuc-1 was observed. A basal level of acetylation is observed in uninfected cell, which increases rapidly on Day 2 post infection followed by subsequent decrease up to Day 17. After this again there is in a slight increase in acetylation at Day 32. This increase could be due to relatively low but active replication of HIV in a subset of cells (Figure-13).

This also indicated the fact that there could be subtle differences and preferential replication within a population of cells, which originated from a single progenitor. Also this could be due to fact that site of integration for virus also plays a major role in level of

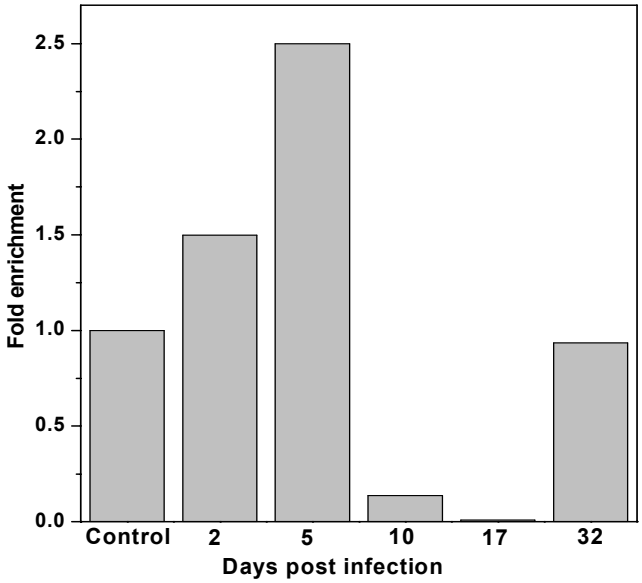

Figure 14: Level of histone 3 acetylation at nuc-1 during the course of infection. The fold enrichment was calculated by normalizing the amount of DNA immunoprecipitated by acetylated H-3 antibody to DNA immunoprecipitated by H-3 antibody. Data shown is mean of two independent experiments.

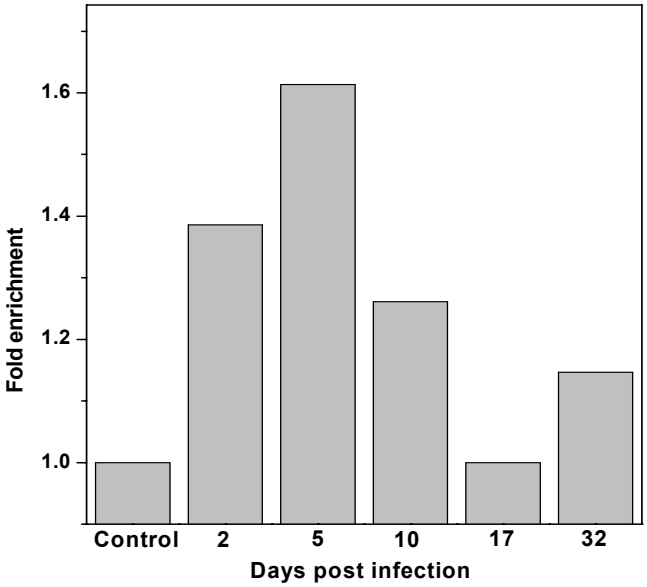

Figure 15: Level of histone 4 acetylation at nuc-1 during the course of infection. The fold enrichment was calculated by normalizing the amount of DNA immunoprecipitated by acetylated H-4 antibody to DNA immunoprecipitated by H-4 antibody. Data shown is mean of two independent experiments.

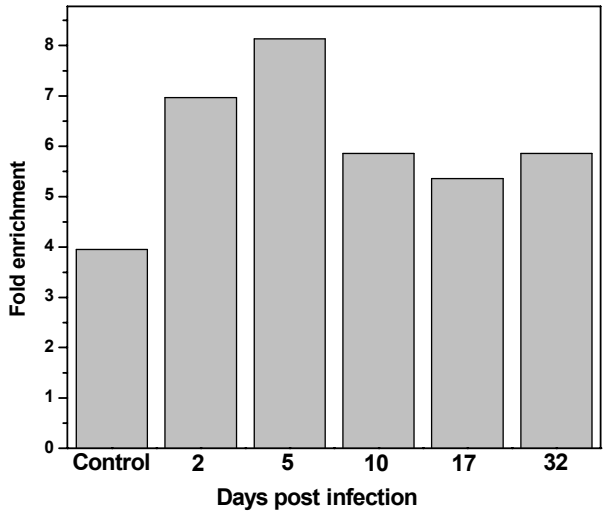

Figure 16: Level of RNA Pol II at LTR during the course of infection. The fold enrichment was calculated by normalizing the amount LTR DNA immunoprecipitated by phosphorylated RNA Pol II antibody to amount TIMM13 DNA in the same sample. Data shown is mean of two independent experiments.

infection. To determine whether transcriptional activation is induced by increase in acetylation of specific histone at the viral promoter *in vivo*, aliquots of chromatin preparation of infected cells were analyzed in parallel by ChIP at different time points. For these purpose antibodies specific for acetylated and native histones were utilized. An increase in acetylation status was observed with increased virus production.

Histone-3 acetylation was estimated using specific antibodies. During infection the Histone 3 of nuc-1 is progressively acetylated till Day 5. Beyond this, levels of acetylation fall significantly at Day 10 and Day 17. After this rapid fall, there is increase in level of acetylation at day 32, which is equivalent to the basal level (Figure-14). A similar pattern in acetylation status at histone 4 of nuc-1 is observed in a similar setup. A basal level of acetylation is observed in uninfected control cells. There is increase in the acetylation status

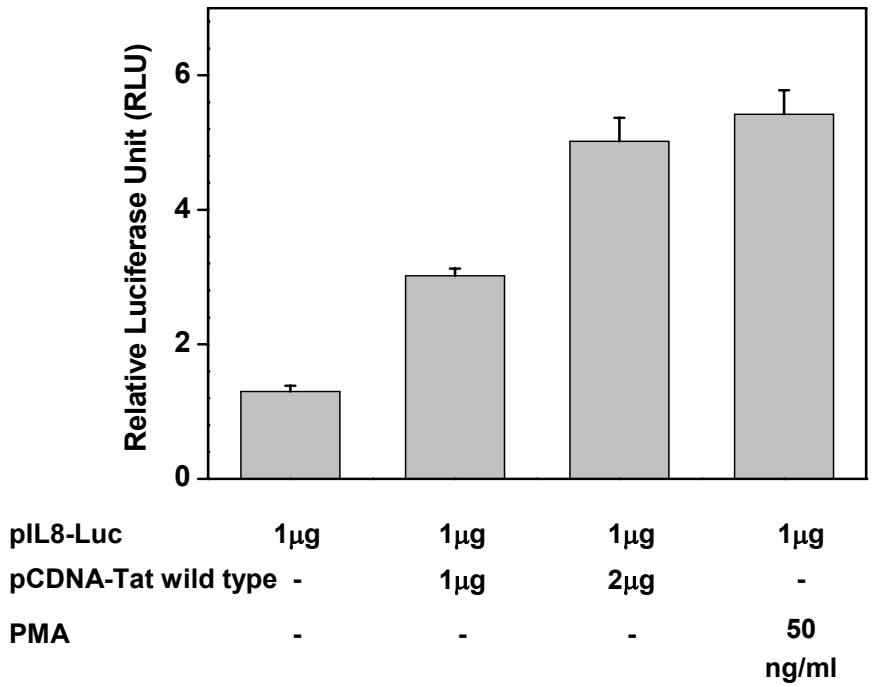

Figure 17: HIV-1 Tat protein activates Interleukin-8 gene promoter driven reporter gene expression. Jurkat cells were transfected with pIL8 Luc (1 µg) reporter vector together with either pCDNA or pCDNA-Tat, or stimulated with 50 ng/ml Phorbol 12-Myristate 13-Acetate (PMA). The normalized data shown represent the mean and SEM of three independent experiments.

followed by return to basal level (Figure-15) the pattern of change at H4 is similar to H3, but is relatively smaller in magnitude.

Recruitment of RNA pol II to promoter is a hallmark of active transcription (58). To gain further insights into transcription initiation status of LTR and its correlation to p24 expression level, qChIP assays were performed with phosphorylated RNA pol II antibodies. A similar pattern of RNA pol II recruitment and viral gene expression in conjunction with Tat occupancy was observed (Figure-16). This also confirms the utility of RNA pol II recruitment as a marker for active transcription of gene in qChIP assay.

4.3.2 Chromatin modulation at cellular genes

4.3.2.1 Interleukin-8

Since, Tat is known to modulate many cellular genes; similar studies were performed to investigate the role of Tat in modulation of cellular gene promoters containing NFκB enhancer sequence. Tat is known to modulate expression of Interleukin-8 (IL-8) gene, an important cytokine, in HIV-1 infection (59-60).

To confirm this modulation, transient transfection of IL-8 promoter luciferase reporter construct (pIL8-Luc) were performed in Jurkat E6 cell line. The cells were transfected with either pIL8-Luc alone or co-transfected with Tat expression construct pCDNA-Tat (Figure-17). An increase in level of reporter gene activity was observed in presence of Tat. This up regulation confirmed that Tat positively modulates IL-8 gene in T cells. To investigate the binding of Tat to IL-8 promoter, ChIP assay was performed with chromatin isolated from infected cells using Tat specific and isotype control antibody. A specific product is obtained

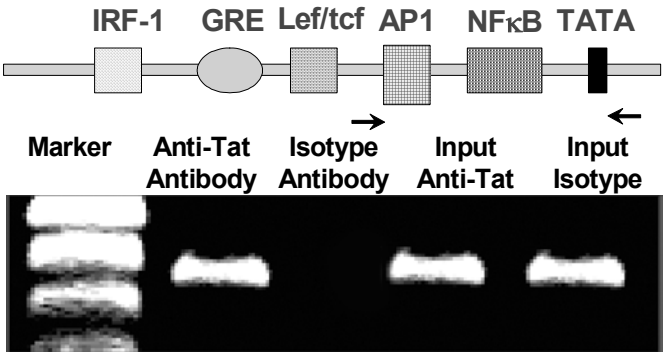

Figure 18: Chromatin immunoprecipitation assay showing interaction of Tat with NFκB enhancer region of Interleukin-8 promoter *in vivo* in HIV-1 infected CEM-GFP cells. Interleukin-8 enhancer region specific PCR product obtained by ChIP assay using antibody against Tat protein and isotype control. Lane 1: Tat IP; Lane 2: isotype control; Lane 3: Tat input control; Lane 4: isotype input control.

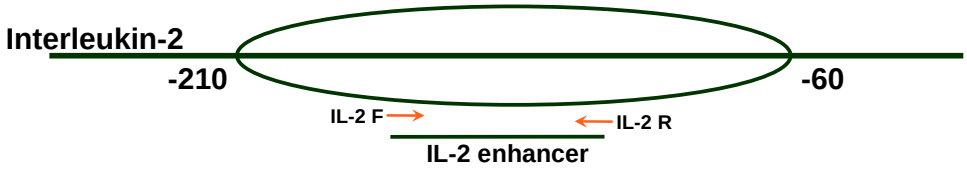

Figure 19: Schematic structure of nucleosome position of IL-2 promoter. The position of nucleosomes encompassing the regulatory region (from nt -60 to -210) of IL-2 promoter is shown. The position of primer pairs used for PCR amplification is indicated by arrows.

with Tat antibody while no product is obtained in isotype control (Figure-18). This confirmed the *in vivo* binding of Tat protein to, IL-8 promoter sequence encompassing NFκB region in infected cells. Since very little information is available regarding the chromatin or nucleosome organization of IL-8 promoter further studies on histone specific modification were rendered difficult.

4.3.2.2 Interleukin-2

IL-2 is one of the important cytokines produced by mature T-cells in response to antigenic stimuli. IL-2 gene regulation is subject to dual control, since it acutely depends on combinatorial signaling through multiple pathways, and can only be activated in a very limited spectrum of cell types (61-75). The regulatory basis for the activation dependence of IL-2 gene expression in T-cells is well-understood. Transcription factor binding sequences of NFAT, NFκB and AP-1 in a 300 bp promoter-proximal region play a key role transcription (75). The IL-2 promoter DNA has the intrinsic ability to assemble a very stable nucleosome core particle, which is both rotationally and translationally positioned between nt -60 and -210 encompassing promoter regions that are critical for inducible expression of the IL-2 gene (75). The IL-2 gene is highly inducible and is rapidly expressed upon T cell activation. Chromatin in vicinity of regulatory region of IL-2 promoter is remodeled upon activation and is a prerequisite for its maximal expression (75).

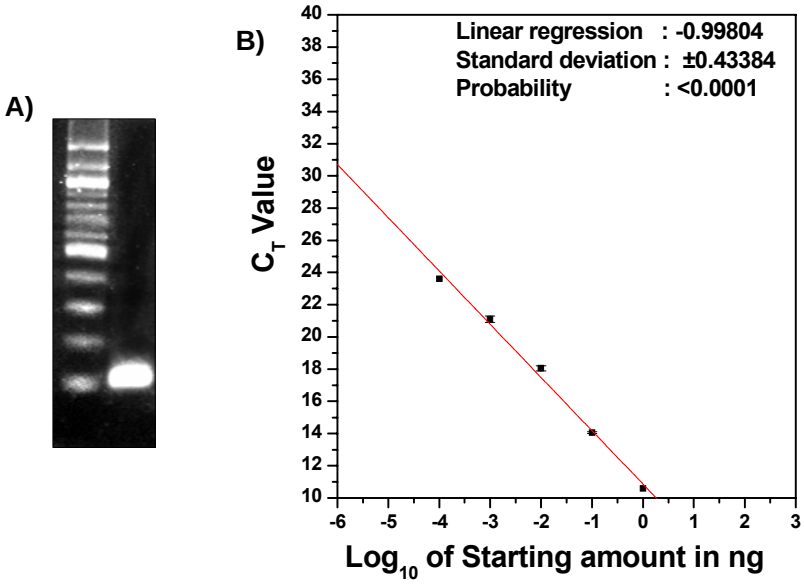

Figure 20: Real time PCR with primers flanking nucleosome of the Interleukin-2 promoter region. A) Primers IL-2 F and IL-2 R were used to amplify the nucleosome region in the promoter. A specific band for amplified product is obtained. **B)** Gel extracted band was used as template to generate the standard curve by real time PCR.

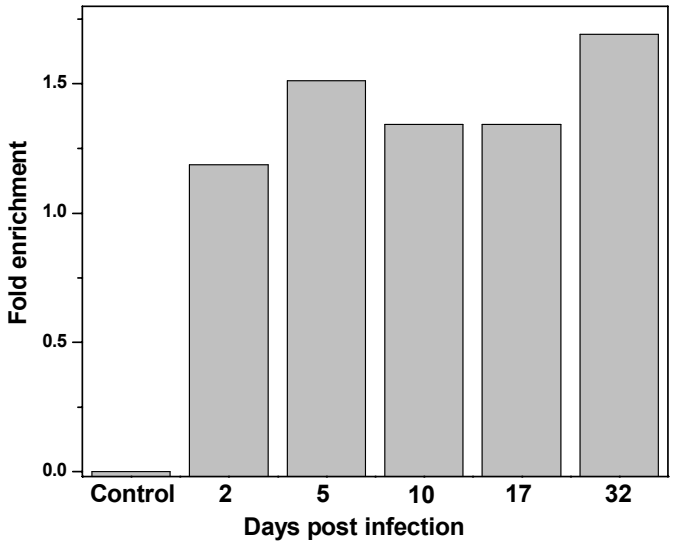

Figure 21: Level of Tat occupancy on Intreleukin-2 promoter in HIV-1 infected cells. The fold enrichment was calculated by normalizing the amount of IL-2 promoter DNA immunoprecipitated by Tat antibody to amount of TIMM13 DNA in the same sample. Data shown is mean of two independent experiments.

Previous results from our lab and others clearly indicate modulation of Interleukin-2 gene expression by Tat protein during infection (49-54). It has been well characterized that this modulation is independent of NFAT induction and NFκB elements play an important role in it. The chromatin architecture in vicinity of transcription start site defines the accessibility of regulatory elements, which also includes NFκB binding sites, to the respective transcription factors. Tat is known to induce chromatin remodeling and can bind to NFκB DNA elements. Studies were performed to find binding of Tat and the pattern of histone modification at IL-2 promoter regulatory region nucleosome during HIV-1 infection.

To investigate the binding of Tat to IL-2 promoter and modulation of chromatin, qChIP assay was performed with chromatin isolated from infected cells using Tat specific antibody. The primers flanking the nucleosome in promoter proximal region of IL-2 (Figure-19) were used to find the levels of Tat occupancy at IL-2 promoter at various time points during the course of infection. Robust amplification was obtained with PCR (35 cycles: 94°C for 30 sec, 60°C for 60 sec, 72°C for 60 sec) using primers IL-2 F and IL-2 R specific for IL-2 promoter encompassing the NFκB enhancer region in presence of 2 mM $MgCl_2$ (Figure-20).

A specific PCR product is obtained with Tat antibody. This confirmed the binding of Tat protein to IL-2 promoter in infected cells. Time dependent variation in Tat occupancy was observed. There is an initial increase followed subsequently by minor decrease in level of Tat binding to IL-2 promoter (Figure-21). Tat modulates chromatin at viral LTR, which leads to increase in gene expression. In order to correlate binding of Tat at IL-2 promoter

with its transcription modulation, further studies were performed to find level of HDAC-1 occupancy and acetylation status of histones at IL-2 promoter.

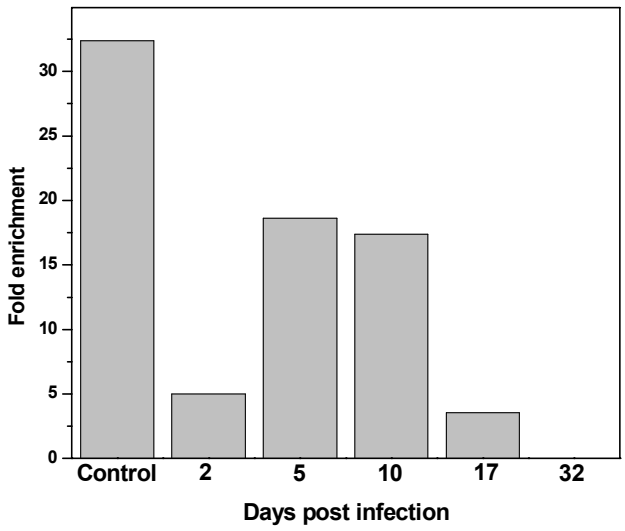

Figure 22: HDAC-1 occupancy at Intreleukin-2 promoter in HIV-1infected cells. The fold enrichment was calculated by normalizing the amount of IL-2 promoter DNA immunoprecipitated by HDAC-1 antibody to amount of TIMM13 DNA in the same sample. Data shown is mean of two independent experiments.

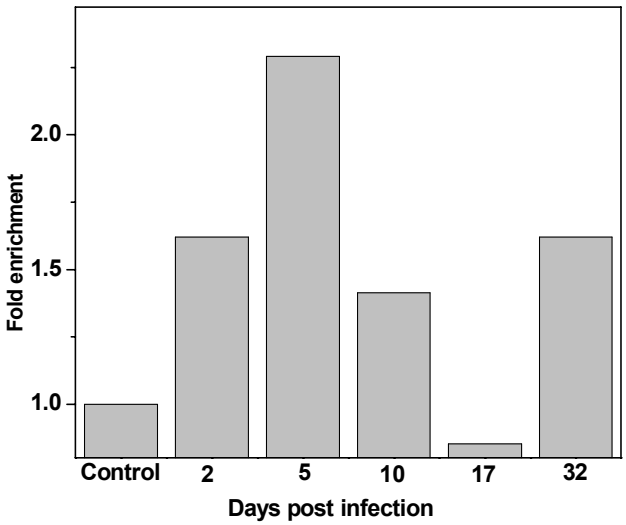

Figure 23: Level of acetylation at Intreleukin-2 promoter during course of infection. The fold enrichment was calculated by normalizing the amount of IL-2 promoter DNA immunoprecipitated by acetylated-lysine specific antibody to the amount of TIMM13 DNA in the same sample. Data shown is mean of two independent experiments.

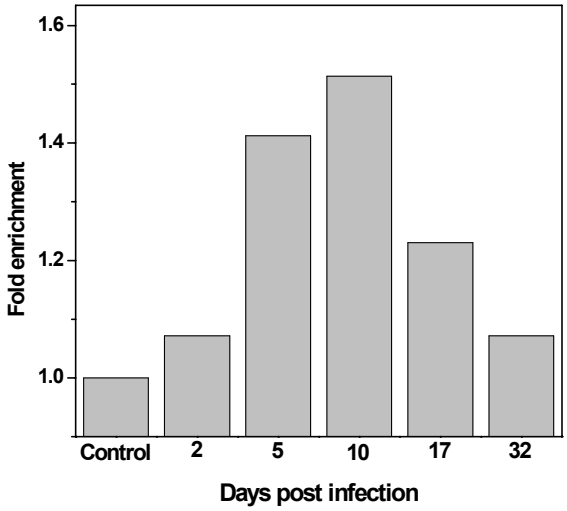

Figure 24: Level of histone 3 acetylation at IL-2 promoter nucleosome during the course of infection. The fold enrichment was calculated by normalizing the amount of DNA immunoprecipitated by acetylated H-3 antibody to DNA immunoprecipitated by H-3 antibody. Data shown is mean of two independent experiments.

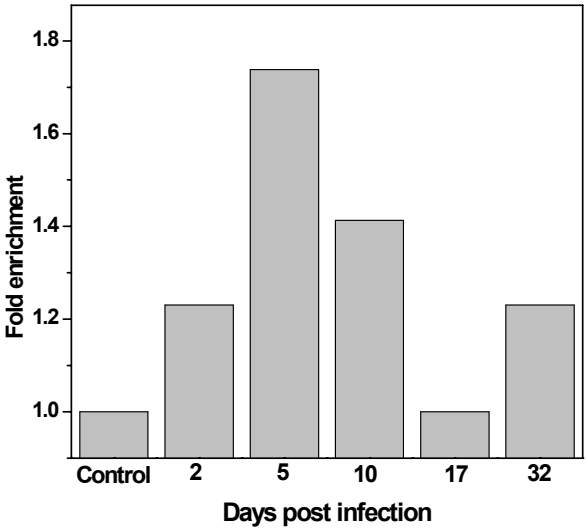

Figure 25: Level of histone 4 acetylation at IL-2 promoter nucleosome during course of HIV-1 infection. The fold enrichment was calculated by normalizing the amount of DNA immunoprecipitated by acetylated H-4 antibody to DNA immunoprecipitated by H-4 antibody. Data shown is mean of two independent experiments.

Earlier a time dependent variation in HDAC-1 levels was observed at LTR during the course of infection. To determine the status of HDAC-1 at IL-2 promoter, qChIP assays were performed as described earlier. Again, a time dependent change in HDAC-1 occupancy of IL-2 promoter was observed. There is a high basal level of HDAC-1 occupancy at the promoter. Upon infection, the level of HDAC-1 is reduced drastically at Day 2. This is followed by increase at in HDAC-1 levels at Day 5 and Day 10 post infection. Beyond this point very negligible amount of HDAC-1 is precipitated in qChIP assay (Figure-22). To find the implication of these variations, level of acetylation on the histone in nucleosome of IL-2 promoter was determined by qChIP assays using pan-acetyl antibody. There seems to be a inverse correlation between acetylation status and HDAC-1 occupancy. An initial increase in levels of acetylation immediately after infection on Day 2, which continues to rise up to Day 5, is observed. There is subsequent decrease in acetylation levels at Day 10 through Day 17 due to increased recruitment of HDAC-1. At Day 32 there is restoration of acetylation levels due to reduced occupancy of HDAC-1 (Figure-23).

To compare whether the pattern of transcriptional activation induced by acetylation of specific histones at the IL-2 promoter is similar to that of LTR, aliquots of chromatin preparation of infected cells were analyzed in parallel by qChIP for H3 and H4 acetylation at same time points. During infection the Histone 3 of nucleosome at IL-2 promoter is progressively acetylated till Day 10. Beyond this level of acetylation there is a slight fall at Day 17 and Day 32. At Day 32 acetylation is almost equivalent to the basal level (Figure-24). A different pattern in acetylation status at histone 4 is observed at same time points. A basal level of acetylation is observed in uninfected control cells. There is a significant

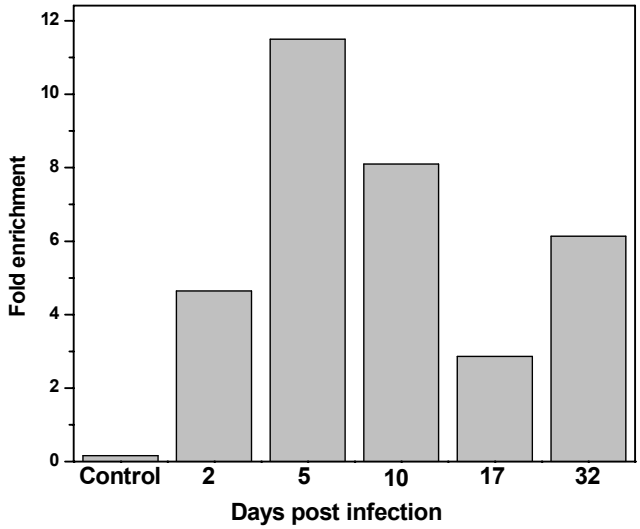

Figure 26: Level of RNA Pol II at IL 2 promoter during the course of HIV-1 infection. The fold enrichment was calculated by normalizing the amount IL-2 promoter DNA immunoprecipitated by phosphorylated RNA Pol II antibody to amount TIMM13 DNA in the same sample. Data shown is mean of two independent experiments.

increase in the acetylation status up to Day 5 after this is acetylation status falls to basal levels (Figure-25). There is significant difference in pattern of changes at histones of LTR and IL-2 promoter. To correlate the histone modification to transcription initiation, RNA pol II qChIP were utilized. A good correlation to acetylation status and transcription initiation was observed (Figure-26).

4.4 Discussion

Gene regulation is a complex process controlled by many cellular factors. These factors are assembled into multiprotein complexes, contributing to specific gene regulation events. Generally transactivation and opening of chromatin is required for gene expression from a promoter. This opening or unwinding is achieved by modification of histone tails, which

makes the nucleosome a basic unit of chromatin (76). There exists a highly dynamic equilibrium of histone acetylation and deacetylation reaction across the chromatin maintained by globally acting enzymes. Targeted acetylases and deacetylases can locally perturb this equilibrium, yet once they are removed; the global activities mediate a rapid return to steady-state level of histone acetylation (76-78).

Given that retroviral DNA is integrated into the human genome and cellular gene expression is controlled by modulation of chromatin structure, thus viral activation and transcription likewise is expected to be modulated by chromatin structure. The replication rate of integrated HIV-1 is largely controlled at the level of transcription. The HIV-1 LTR present at both ends of the integrated viral genome contains *cis*-acting elements necessary for transcription initiation from the 5' LTR and for polyadenylation of the viral transcript in the 3' LTR. The proviral DNA is organized into a higher order chromatin structure *in vivo*, which regulates viral expression by restricting access of the transcriptional machinery to LTR. HIV-1 gene expression can vary from 1-70 fold based on site of integration and chromatin structure of LTR and its vicinity (79). Regardless of the site of integration, the integrated 5′ LTR of HIV-1 is packaged by two nucleosomes separated by a distance of 265 base pairs (47). Although the precise nucleosome position of integrated LTR is well characterized, there is little data about how this primary structure is folded into the chromatin fiber or other secondary structure. There is strong evidence that chromatin environment influences basal HIV-1 gene expression. While the specific roles of and interplay between viral and host proteins have not been fully elucidated, numerous reports indicate that HIV-1 retains the ability for self-regulation via pleiotropic effects of its viral proteins.

Previous reports described specific disruption of nuc-1 as a result of histone modification following HIV-1 activation in latently infected cells (43, 44) and provide a definite role for chromatin remodeling in viral expression. Albeit, all these studies utilized cell lines which harbored a defective replication incompetent virus which requires external stimuli for reactivation. No reports on chromatin modulation studies involving actively replicating virus are available. The model of CEM-GFP cell line infected with HIV-1 NL 4.3 utilized in this study provides opportunity to investigate chromatin status in this scenario.

Tat is a key regulatory protein of HIV-1. It is expressed early even before the integration (79-81) and stimulates the transcriptional initiation and elongation by RNA polymerase II (RNA Pol II). Tat is involved in both transcription initiation and elongation, and also plays an important role in chromatin modulation by recruiting various factors to the promoter. Role of Tat in remodeling of LTR chromatin in a latently infected cell line has been reported recently (42). There is an increase in H3 and H4 acetylation at nuc-1 during activation of latent promoter by Tat and also PMA, a known activator of T-cells (42). Most of the earlier studies have focused on effect of Tat and modification of specific histones during reactivation of latent virus. The focus of this work was to study the levels of Tat and HDAC-1 recruitment at LTR and analysis of histone modification immediately after infection and follow up till the cells have established infection.

It is well documented in literature that Tat can recruit HATs and dislodge the HDACs from the LTR, which leads to histone acetylation. Also, role of HDAC-1 in modulation of gene expression for various cellular genes as well as LTR is well established (48). Hence, the occupancy of HDAC-1 on nuc-1 of LTR and its effect on histone acetylation and gene

expression were also investigated. A large increase in global acetylation levels at LTR is observed immediately on Day 2 post infection. A subsequent fall in acetylation status and increase in HDAC-1 recruitment is observed. It appears that there exist a fine control and close interplay between recruitment of HDAC-1 and acetylation status of nuc-1, indicating its dynamic role in chromatin-mediated regulation of gene expression from LTR. An excellent correlation of this interplay with expression of viral genes is observed. The expression level increase initially and peaks at Day 5 after which it is maintained at low but significant levels during further progress of infection.

Acetylation of Histone-3 and Histone-4 is hallmark of active transcription. Although acetylation of both of these histones is always associated with active transcription, a definite role for subtle variation in pattern of modification has been reported (82). To determine the effect of HDAC-1 recruitment on acetylation of specific histone, similar assay was carried out using antibodies directed against histone-3, acetylated histone-3, histone-4, and acetylated histone-4. We observed variations in H-3 modification as compared to H-4 specifically during middle of the time course of infection. A basal level of H-3 and H-4 acetylation is observed in uninfected cells that increase initially upon infection. There is deacetylation observed at H-3, which falls below basal level on Day 17 but not at H-4, which is reduced only to a lesser extent. On further infection there is restoration of acetylation at H-3 and further deacetylation at H-4 to basal level by Day 32. Histone acetylation is always correlated with increased expression. The pattern of variation in histone acetylation at LTR is reflected at both gene expression and viral production as analyzed by GFP expression and p24 antigen production.

Contrary to studies with reactivation of latent virus in chronically infected cell lines, a clear and specific recruitment of HDAC-1 was observed in the present studies. Initial studies have underscored an important role for Tat in reactivation of virus from latency (83). We observed binding of Tat to LTR which correlated with activation status of viral gene. It could be hypothesized that Tat plays a crucial role in initiation of viral expression during early phase. But later when a certain threshold of gene expression is achieved multiple mechanisms take over to maintain sustained infection.

Tat has been shown to induce chromatin remodeling by recruiting histone acetyl transferases (HATs) such as p300 and p300/CBP associated protein P/CAF to the chromatin, which results in histone acetylation and acetylation of itself (44-48). Tat could derepress the integrated HIV-1 chromatin structure, aiding in activation of transcription of integrated transcriptionally silent HIV-1 promoter. Hence, it could be possible that that Tat continuously recruits HATs to LTR and maintain a minimal expression by binding to promoter. This may result in shortage of cofactors required for transcription and other host factors trying to suppress the expression by recruiting HDAC-1. This leads to periodic variation in acetylation and helps in continuation of viral expression as well prolonging the life of host cell.

Further studies were carried out on cellular gene promoters to gain further insight into binding and universality of Tat at a global level. Interleukin-8 gene, which is modulated by Tat in infected cells and not known to encode any TAR like mRNA structure, was used initially as model system for these studies (59, 60). Transient transfection based reporter assay confirmed the up-regulation of IL-8 promoter by Tat. The binding of Tat to NFκB enhancer region in IL-8 promoter was confirmed using ChIP assay. It could be noted that

IL-8 gene promoter does not bear any resemblance to LTR. Tat binding and modulation of IL-8 promoter confirms its recruitment and binding to NFκB enhancer is independent of any other Tat interacting transcription factors.

Since, the nucleosome organization map for IL-8 promoter is not well characterized further studies relating to histone modification were rendered difficult. Therefore, studies were performed with Interleukin-2, a cytokine gene with well-characterized nucleosome map (75) and modulated by Tat during infection (49-54). Tat binds to IL-2 promoter chromatin in infected cells. This proved that Tat could bind NFκB enhancer sequence in many cellular genes and regulate them. Further studies were then performed on chromatin modulation at IL-2 promoter. In contrast to HIV-1 LTR, a high level of HDAC-1 occupancy and low level of acetylation was observed at IL-2 promoter in uninfected cells. This indicates that, as reported previously, chromatin remodeling could play a major role in modulation of IL-2 promoter. Upon infection, a significant increase in acetylation and decrease in HDAC-1 is observed. HDAC-1 is again recruited to IL-2 promoter, which results in decrease in acetylation. The levels HDAC-1 are significantly lesser when compared to uninfected cells. A basal level of acetylation status was observed in both H-3 and H-4, which increases upon infection. The acetylation of H-3 is maximal at Day 10 and this subsequently returns to basal level, while H-4 follows a pattern similar to global levels, where acetylation initially rises up to Day 5 and subsequently varies according to HDAC-1 occupancy. This suggests that H-4 modifications plays major role in control of IL-2 gene expression. In previous reports, nucleosome remodeling was observed at IL-2 promoter during it's over expression upon stimulation with other activating signals (75). These

reports and our results indicate that H-4 modifications plays major role in control of IL-2 gene expression.

A deviation in acetylation pattern is observed between LTR and IL-2 promoter. There is a decrease in acetylated H3 at LTR much below the basal level on day 17, while H3 at IL-2 promoter displays much higher level of acetylation. Acetylation levels at H4 follow the same profile both at LTR and IL-2 promoter. This suggests the fact that though H-3 and H-4 acetylation is marker for activation, there is a considerable promoter specific variation in modifications. A specific histone code hypothesis was proposed on the mechanism by which chromatin modification exerts its influence on gene regulation (84). But, recent data available through yeast genetic studies show that it may not be as complicated as believed earlier (85). In last few years although a lot of information has been generated in respect to chromatin modification, there are many unresolved questions yet to be answered to completely understand the histone code. It appears from our data that gene expression always correlates with acetylation and suggests involvement of a gene specific code.

The regulatory language of the genome seems to have the complexity and features similar to that of spoken language. Rather than universal, the promoter specific chromatin structure or its modulation identified in this study is likely to be cell type and stimuli specific. Activators, chromatin-modifying enzymes, and basal transcription factors unite to activate genes, but are recruited in a precise order to promoters. Only an accurate and predefined order of events which lead to recruitment of factors can activate transcription of a gene (86). The transcription is temporally arranged event in which the activation of genes occur at precise moments such that protein product is present at particular time point. Proteins are required at proper place and time to perform its functional role which

otherwise leads to deregulation. No functional event inside the cell occurs by chance and has definite temporal function dictated by its own different needs. We observe a temporal correlation of Tat recruitment and HDAC-1 occupancy with gene expression at LTR as well as IL-2 in HIV-1 infection. Finally the result obtained with the present model system of HIV-1 infection indicates that gene regulation in infected cells is much more complex than is currently appreciated.

4.5 References

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List of publications

1) **Dandekar, D. H.**, Ganesh, K. N., and Mitra, D. (2004) HIV-1 Tat directly binds to NFκB enhancer sequence Role in viral and cellular gene expression. *Nucleic Acids Res*., **32:** 1270-1278.

2) **Dandekar, D. H.**, Kumar, M., Ladha, J. S., Ganesh, K. N., and Mitra, D. (2005) A quantitative method for normalization of transfection efficiency using enhanced green fluorescent protein. *Analytical Biochem*., **342:** 341-344.

3) **Dandekar, D. H.**, Ganesh, K. N., and Mitra, D. (2005) HIV-1 Tat interacts as a dimer with NFκB enhancer DNA sequence: A Fluorescence anisotropy and Isothermal titration calorimetry study. (manuscript under preparation)

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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).

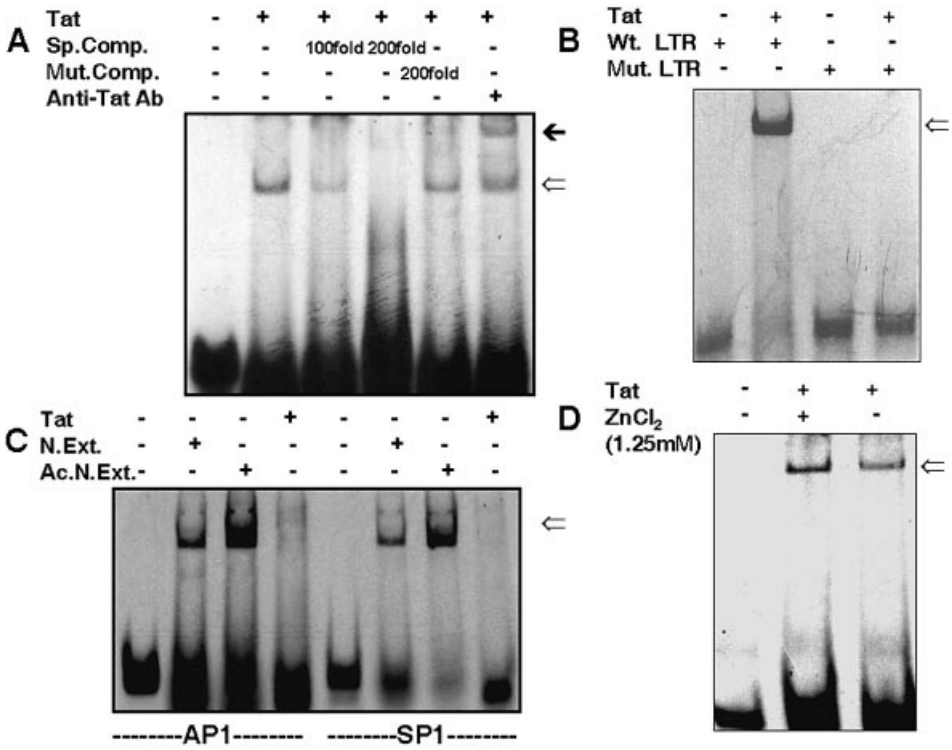

Figure 2. Tat interacts specifically with NFκB enhancer sequences. (**A**) Gel shift assay using NFκB consensus oligonucleotide as labeled probe and cold specific and mutated oligonucleotide as competitors. The sequences are given in Table 1. Lane 1, free probe; lane 2, Tat protein; lane 3, 100-fold 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

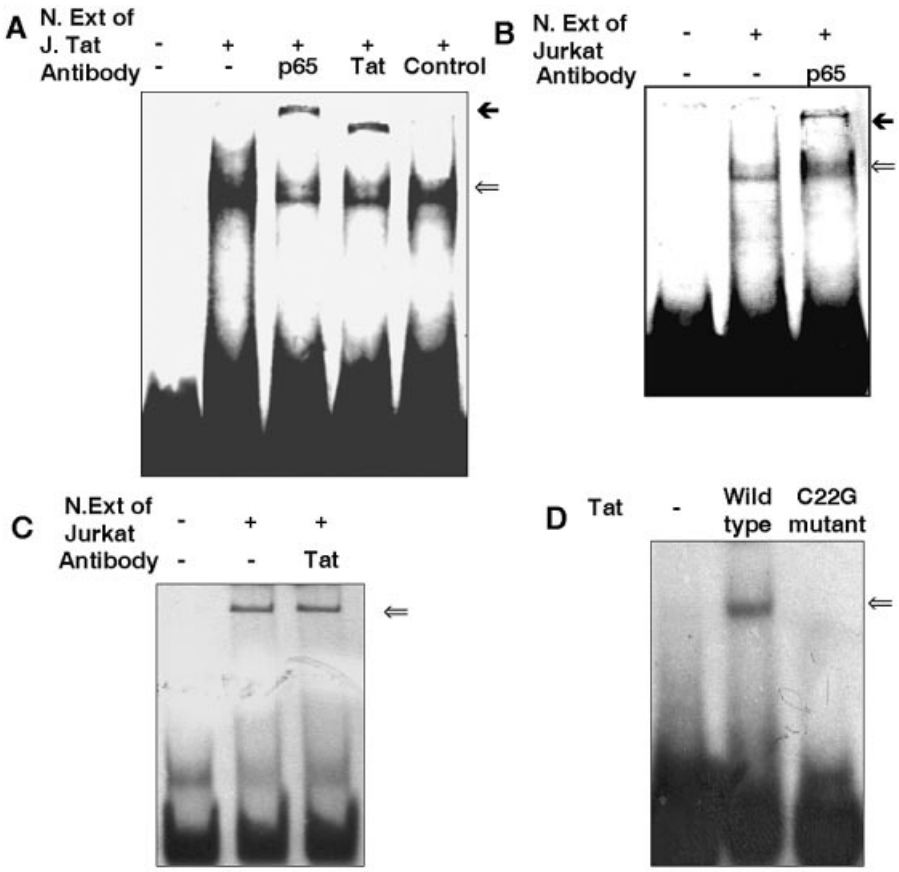

Figure 3. LTR-NFκB enhancer interacts with purified Tat protein and endogenously expressed Tat but not with C22G mutant Tat protein. (**A**) Gel shift assay showing binding of endogenously expressed Tat in Jurkat-Tat nuclear extract to LTR NFκB oligonucleotide. Lane 1, free probe; lane 2, nuclear extract 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

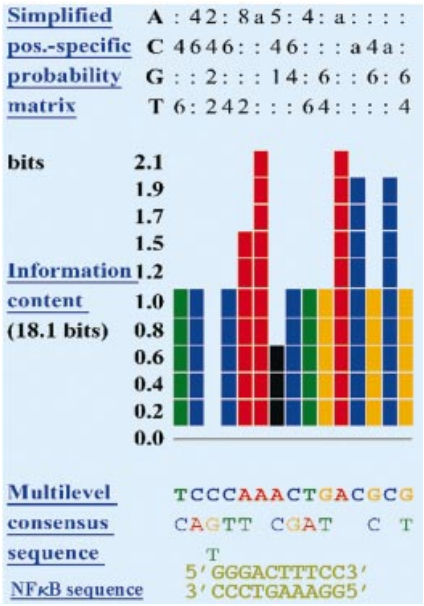

Figure 4. Position-specific matrix generated by MEME software analysis. DNA sequences obtained by oligonucleotide library screening were analyzed as described in the text. The matrix shows the bit score for the occurrence of a particular nucleotide in the motif, total bits score as calculated and consensus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

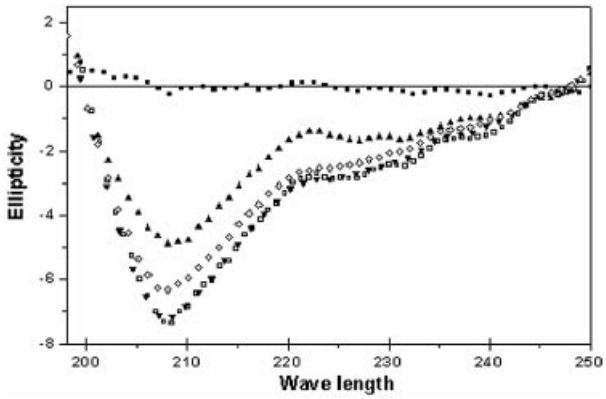

Figure 5. CD spectra of Tat protein in the presence of LTR-NFκB oligonucleotide: solid triangles (down), CD spectrum of Tat protein, 30 μg/ml in PBS pH 7.4; solid triangles (up), change in CD signature of Tat protein in the presence of LTR-NFκB oligonucleotide, 0.1 nmol; open diamonds, change in CD signature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

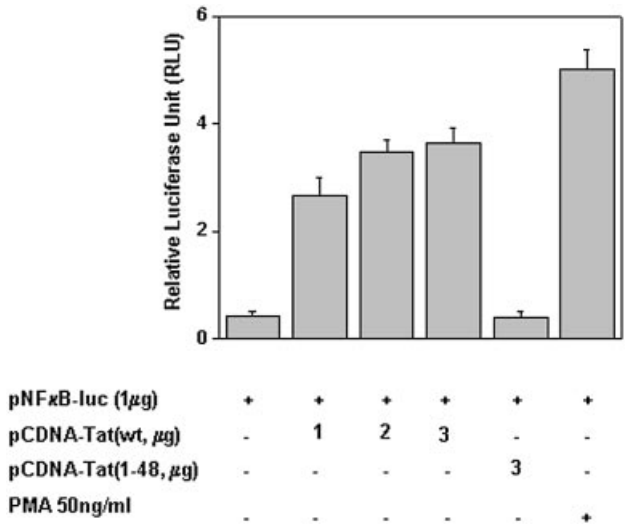

Figure 6. HIV-1 Tat protein activates NFκB enhancer driven reporter gene expression. HEK 293T cells were transfected with pNFκB-luc (1 μg) reporter vector (Stratagene, USA) together with either pCDNA-Tat or pCDNA-Tat (1–48), or were stimulated with 50 ng/ml PMA. pEGFPN1 vector (Clontech, USA) was co-transfected 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

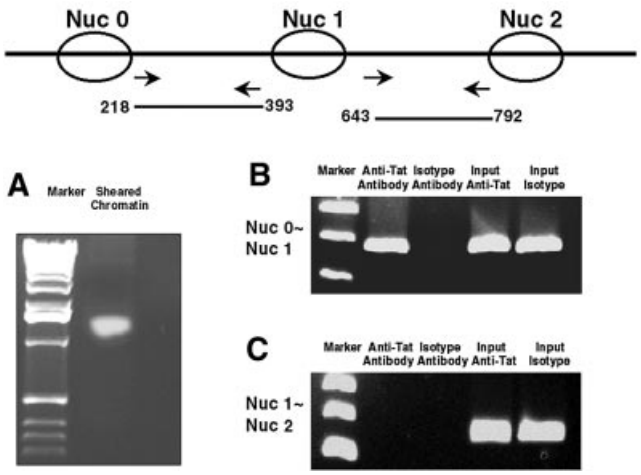

Figure 7. CHIP assay showing interaction of Tat with the enhancer region of the integrated LTR *in vivo* in HIV-1-infected CEM-GFP cells. HIV-1 NL4.3-infected CEM-GFP cells were cross-linked by 1% formaldehyde followed by quenching with 125 mM glycine. Cells were washed and the pellet was lysed, followed 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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Notes & Tips

A quantitative method for normalization of transfection efficiency using enhanced green fluorescent protein

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Transient-transfection-based reporter assay is an excellent model system to study the function of promoters. Cotransfection of a second reporter expressing β-galactosidase, *Renilla* luciferase, or green fluorescent protein (GFP)[1] has been widely used to normalize the inherent variation in transfection efficiency. Existing methodology of GFP-based normalization involves either visual counting or flow cytometric analysis, both of which are laborious and time consuming. We report here a simple and rapid fluorometric quantitation of enhanced green fluorescent protein (EGFP) for normalization of transfection efficiency. The method described is robust, requires reduced handling, and is adaptable to high-throughput screening format.

Introduction of foreign genes into mammalian cells or transfection is of great interest both for basic biological research and for gene therapy and is routinely practiced in molecular and cell biology laboratories. Regulatory functions of promoters are normally characterized by transient-transfection-based reporter gene assay. One of the basic drawbacks of such assay is unequal efficiency of transfection. Internal control reporter vectors are widely used in transient transfection assay to monitor inherent variation in transfection efficiency between experiments and/or to normalize transcriptional activity. A number of methods that utilize simultaneous transfection of reporter and internal control plasmid have been described [1]. There are a number of in vitro reporter genes, such as secreted alkaline phosphatase, β-galactosidase, firefly luciferase, and chloramphenicol acetyltransferase, available for use in transfection studies and quantifying transfection efficiencies [1]. The dual-luciferase assay system based on firefly luciferase reporter and normalization with *Renilla* luciferase from Promega, USA is one of the most popular and widely used commercial systems currently available for normalization. A number of published reports have demonstrated a drawback of measuring the cumulative enzymatic activity of gene product to determine transfection efficiency [2,3]. The GFP from the jellyfish *Aequorea victoria* has become an important reporter for monitoring gene expression and protein localization in a variety of cells and organisms [4]. GFP expressed in eukaryotic cells yields green fluorescence when cells are excited by ultraviolet (UV) or blue light. Many variants of this wild-type GFP with improved stability and spectral properties are now available. The EGFP is a highly fluorescent and stable mutant of GFP [4] and is being used currently in a variety of biological applications such as measurement of gene expression and cell labeling and protein labeling localization studies [5]. The chromophore in EGFP is intrinsic to the primary structure of the protein, and fluorescence from EGFP does not require additional cofactors, substrates, or additional gene products. Hence it serves as an excellent tool for noninvasive study of reporter gene expression in cells. Flow-cytometry-based quantitation [6] and microscopic visualization [7] have been widely used for EGFP expression studies but a rapid quantitative assay has not been

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[1] *Abbreviations used:* GFP, green fluorescent protein; EGFP, enhanced GFP.

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available. Both these techniques are useful but are time and labor intensive particularly in the case of multiple samples and cannot be used in high-throughput analysis. Although fluorometric quantitation of GFP has been reported previously in few cell-based assays [8], its application in quantitative analysis of GFP expression in transient transfection assays has not been studied. Here we show quantitative analysis of GFP expression by fluorimetry in transient transfection experiments and its use in normalization of transfection efficiency in reporter assays.

In the present study we have used two reporter systems: a firefly luciferase reporter downstream of HIV-1 LTR promoter (pLTR-Luc), constructed by subcloning the LTR from pU3RIII [9] into pGL3-basic (Promega), as a model system to test the regulatory role of the promoter to be analyzed and a plasmid pEGFP-N1 (Clontech, USA) that encodes EGFP under the control of CMV promoter as the second reporter. We have also used HIV-1 transactivator protein Tat encoding expression vector pCDNA-Tat, which has been described previously [10].

HEK 293T cells obtained from the National Centre for Cell Science cell repository were plated at density of 6×10^5 cells/well in a six-well plate and allowed to adhere to plate; subsequently, plasmid vectors were transfected with Lipofectamine-2000 (Invitrogen, USA) according to manufacturer instructions. The cells were cotransfected with reporter vectors pLTR-Luc and pEGFP-N1 along with HIV-1 Tat expressing vector pCDNA-Tat or empty vector pCDNA 3.1 to study the use of EGFP as a second reporter. Also plasmid expressing *Renilla* luciferase (pRL-SV40) under the control of SV40 promoter (Promega), was cotransfected with the above plasmids in some experiments to compare EGFP and *Renilla* luciferase as reporters.

Cells were harvested 36 h posttransfection and washed twice with phosphate-buffered saline, 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.4 mM KH_2PO_4, pH 7.3. The cells were then lysed in 25 mM Tris-phosphate, pH 7.8, 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetra acetic acid, 10% glycerol, and 1% Triton X-100. This buffer is also available from Promega as Luciferase Cell Culture Lysis Reagent and will be henceforth termed CCLR. The lysate was centrifuged at 12,000*g* for 2 min at 4 °C and the clarified supernatant was used for measurement of both GFP and luciferase activity. All measurements were recorded on Fluoroskan *Ascent* FL microplate recorder (Labsystems, Finland) which can be used for both fluorescence and luminescence measurements. Prior to estimation of luciferase, the EGFP fluorescence was estimated for the samples in the wells of black combiplate (Labsystems) with filter set at excitation 485 nm and emission 510 nm. The luciferase activity was then measured in the same plate using the substrate from a single luciferase assay kit (Promega) according to manufacturer's instructions. The luciferase activity was normalized to EGFP fluorescence units (signal strength). This also prevented sample alteration during processing.

Initially, to determine the utility and sensitivity of EGFP in transfected cells, we transfected various amounts of pEGFP-N1 (0–1 μg) in 293T cells using Lipofectamine-2000 in six-well plates. The cells were lysed in CCLR; the clarified lysates were used to quantify fluorescence using Fluoroscan *Ascent* FL as described earlier. An equal amount of protein, as estimated by Bio-Rad protein assay kit II (Bio-Rad, USA), was used for quantitation of fluorescence. A linear correlation of the fluorescent signal, normalized to the amount of protein in the lysate, was observed with increasing amounts of plasmid DNA (Fig. 1A). A very good signal was observed with as little as 250 ng of pEGFP-N1. This demonstrated the utility of EGFP plasmid as reporter vector in transfection studies. The transfections were repeated several times to confirm the observation. The sensitivity and linear range of response for EGFP was studied using lysate of cells transfected with 1 μg of pEGFP-N1. A linear response of signal was found with increasing concentration of protein up to 250 μg (Fig. 1B). The signal was also detectable in very dilute samples. This demonstrated the utility of EGFP at very low expression levels. This is particularly useful in cells, which are refractory to transfection and exhibit poor expression of reporters.

To determine whether EGFP can be used as internal standardization control we cotransfected HEK 293T cells with pEGFP-N1 and pLTR-Luc along with pCDNA 3.1 or the HIV-1 transactivator Tat expression vector pCDNA-Tat. The cells were harvested, lysed in CCLR, and then assayed for fluorescence and firefly luciferase activity in the same sample. Thus the method afforded internal standardization controls for both transfection efficiency and sample loss during processing. While similar levels of EGFP fluorescence units were observed for both samples, around 65-fold increase in luciferase units was observed in the presence of Tat, a specific transactivator of HIV-1 LTR. (Fig. 2A). The normalization of transfection efficiency was obtained by dividing arbitrary light units with fluorescence counts. To directly compare the *Renilla* luciferase and EGFP reporter as internal controls, expression vector pRL-SV40 was also cotransfected along with other plasmids mentioned above. The cells were harvested after 36 h and assayed for EGFP fluorescence followed by assay of firefly and *Renilla* luciferase using the dual-luciferase assay kit (Promega). Similar expression levels for normalized transactivation were observed using both *Renilla* luciferase and EGFP as internal controls in the same experiment (Fig. 2B). Thus the present quantitative assay for green fluorescent protein can be used in place of *Renilla* luciferase, which will reduce not only the cost of the assay but also the time required.

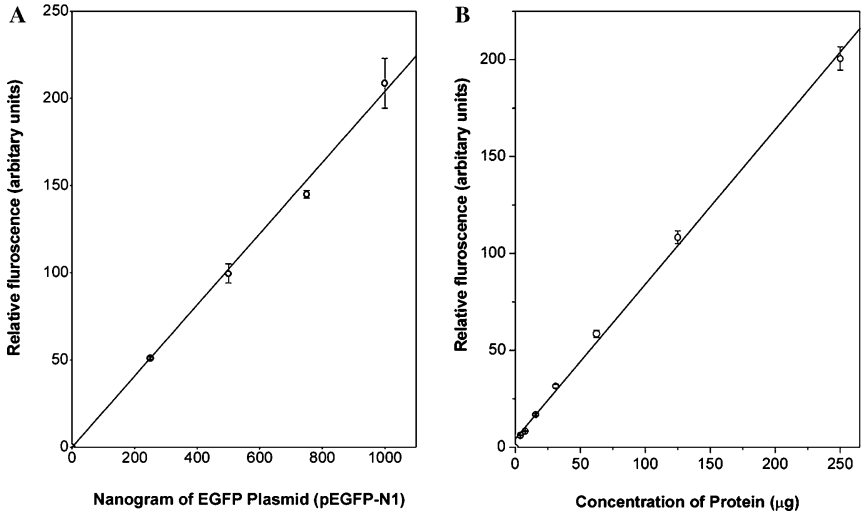

Fig. 1. Green fluorescent protein as sensitive reporter molecule in cell lysate. 293T (6×10^5) cells were transfected with plasmid pEGFP-N1. The cell lysate was centrifuged at 12,000*g* for 2 min at 4 °C and the clarified supernatant was used for measurement of GFP using Fluoroscan Accent FL microplate reader as described in the text. (A) Sensitivity of EGFP using varying amount of pEGFP-N1 (0–1 μg) in a six-well plate. An equal amount of protein (200 μg) was used for quantitation of fluorescence. (B) The sensitivity and linear range of response for EGFP using different concentrations of clarified cell lysate protein transfected with 1 μg of pEGFP-N1. The data shown represent means ± SE of three independent experiments.

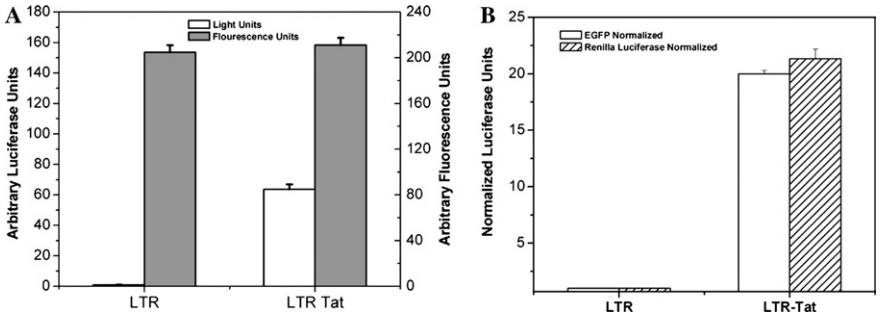

Fig. 2. Green fluorescent protein as an internal control reporter to normalize inherent variation in transfection efficiency. The cells were lysed and total protein extract was used to measure the promoter activity. The lysate was centrifuged at 12,000*g* for 2 min at 4 °C and the supernatant was used for measurement of both GFP and luciferase activity. (A) Firefly luciferase reporter assay using single luciferase assay kit (Promega) after GFP quantitation in a cotransfection of pEGFPN1 (1 μg) with LTR reporter (1 μg) and Tat (1 μg). (B) Comparison of normalized promoter activity using dual-luciferase assay kit (Promega) containing firefly and *Renilla* luciferase with firefly luciferase and EGFP. The data shown represent means ± SE of three independent experiments.

We have also analyzed the luminescence using the Luclite kit (Perkin-Elemer life Science, USA) with a TopCount microplate counter (Packard, USA), after quantifying the fluorescence in the wells of an Opti-plate (Packard Bioscience, USA) and the results are reproducible as before. The same results were also obtained when the Luclite reagent was used directly in place of CCLR for lysis and quantitation of EGFP in Fluoroskan *Ascent* FL followed by luciferase quantitation in the TopCount microplate counter. Finally similar results were also observed in transfected Jurkat and Hela cell lines, indicating possible use of the methodology in a wider scenario (data not shown). This assay hence can be adapted for high-throughput screening of transfected cells.

With reporter proteins and detection systems constantly being improved, luminescent and fluorescent assays are becoming more prevalent because of the ability to visualize reporter activity inside cells. Reporters such as GFP and luciferase, which provide a highly sensitive but nondestructive way of monitoring gene transfer and expression, are becoming increasingly popular. Reporters such as β-galactosidase, GFP, and *Renilla* luciferase have been commonly used for normalization with firefly luciferase but existing methodology requires time-consuming processing and analysis at least for GFP and β-galactosidase. While the use of the *Renilla* luciferase reporter gene as an internal control is adequate for most experimental conditions, increased cost of this system limits its applicability to large-scale high-throughput use. In addition, the buffer system available for such a dual-luciferase kit may compromise for the dual applicability and hence may not be optimal for activity of either reporter. Recent reports have demonstrated

unexpected sensitivity of this system to certain reagents, stimuli, and cellular factors leading to erroneous interpretation of results [2,3]. This has necessitated search for reliable alternatives.

The requirement of additional steps, reagent, and considerable time is avoided using fluorometric quantitation of EGFP for normalization. The fluorescence of EGFP is intrinsic due to the presence of chromophore in the protein, consisting of an imidazolone ring structure formed by posttranslational cyclization reaction and oxidation reaction involving a tri-peptide in primary structure. This fluorescence is free from the cellular environment and can be visualized and quantified [11,12]. The method described here is reliable for normalizing transient reporter assays under a variety of conditions, is robust, and can be used for high-throughput screening. The advantage of this assay is high sensitivity and selectivity, simpler manipulation procedures (e.g., reduced purification or cell lysis), and adaptability to large-scale (e.g., high-throughput screening) measurements.

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