Characterization of c-Myc-Mediated Repression of the Human Immunodeficiency Virus Type 1 Promoter

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ABSTRACT

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The effect of trans-acting factors on cis-acting DNA elements on the HIV-1 promoter is the principal determinant governing transcriptional activation or repression. Few host factors that limit viral transcription and contribute to stable, nonproductive infections have been described. C-myc is a nuclear phosphoprotein that we have shown can negatively regulate HIV-1 promoter expression and viral replication in CD4+ T-lymphocytes. The mechanism implicates c-myc in a direct role. The electrophoretic mobility shift assay demonstrated that c-myc could specifically recognize the HIV-1 initiator element and linker scanning mutagenesis of the LTR confirmed the loss of c-myc-mediated repression in the absence of this region. C-myc can also recognize E box motifs. This element is present within the initiator region and has been demonstrated to be occupied by the c-myc related protein, USF which stimulates HIV-1 transcription. Results from co-expression studies indicated that c-myc mediated repression did not occur through direct binding to the E box and competition with USF. Moreover, c-myc appears to cooperate with initiator binding proteins, YY-1 and LSF to additively reduce gene expression. Co-immunoprecipitation experiments indicated that these cellular factors are capable of interacting *in vivo* and in the absence of the HIV-1 long terminal repeat. This suggests that c-myc, YY-1, and LSF are capable of forming a multiprotein complex and preassembling prior to binding to the initiator element.

Thus, c-myc negative regulation of HIV-1 transcription offers one potential explanation for the emergence and maintenance of latent proviral reservoirs.

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Above all, I would like to thank my mother for the enormous sacrifices she has made and for being an endless source of motivation, and inspiration. I dedicate this work to her.

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INTRODUCTION

1.1 The Human Immunodeficiency Virus Type-1

HIV-1 combines persistent infection with immunodeficiency. Infection is characterized by a progressive decline in both numbers and function of CD4+ T helper lymphocytes which play a central role in coordinating immune responses. Ultimately, the weakened immune system is overcome by replicating virus and the fatal syndrome known as AIDS develops (1,2,21,56,61).

The first reported case of HIV-1 was documented in 1981 (2,61). By 1992, HIV-1 was the leading cause of death on a global level. Today, 36 million people have been infected and 15,000 people continue to be infected daily worldwide. Despite intensive efforts, an HIV-1 vaccine has not been achieved. Presently, the most effective treatment strategy is limited in scope to interference with active viral replication and has no effect on the subpopulation of host cells stably, but nonproductively infected with HIV-1 (14,51,53,71). In addition, a cumbersome drug regimen, and side effects have led to poor compliance. Thus, initial optimism that highly active antiretroviral therapy could prolong life expectancy and eventually eradicate HIV-1 has been tempered by drug resistance, and viral relapse when therapy is discontinued. Increased basic understanding of the virus and the pathogenic process it induces can provide clues that will hopefully lead to novel protective and therapeutic interventions that are so urgently needed.

1.2 Properties of the HIV-1 Virion

The human immunodeficiency virus is a retrovirus of the lentivirus subfamily (1,24,56,61,87,118,122,126). It is an icosahedral sphere approximately 80 to 100nm in diameter with a unique three layered structure (1). Retroviruses differ from other classes of viruses in several respects:

- They possess the only diploid genome
- It is the only viral RNA synthesized and processed by the transcriptional machinery of the host cell
- The RNA is associated with a specific tRNA-lysine to prime replication
- It is the only positive sense, single stranded RNA genome that does not serve as a messenger RNA soon after infection (1).

All lentiviruses are enveloped by a lipid bilayer derived from the host cell plasma membrane and embedded with cellular proteins including the major histocompatibility complex I, II (MHC), actin, and ubiquitin (122). The 72 peplomers projecting from the envelope are oligomers of two noncovalently linked glycoproteins that are cleaved from a gp160 precursor by cellular proteases (1,63). The surface protein, gp120 is the receptor binding ligand and is the most highly glycosylated viral protein that functions as a defense mechanism from neutralizing antibodies. It is anchored to the virus via interactions with the hydrophobic transmembrane protein, gp41. The inner membrane is a myristoylated matrix composed of 2000 copies of protein p17. The viral core consists of a cone shaped capsid comprised of p24 which represents the most abundant viral protein. The capsid encloses two copies of the unspliced, 9.8Kb genome which is stabilized as a ribonucleoprotein complex consisting of nucleocapsid proteins, p9, p6 and the enzymes

reverse transcriptase (RT), ribonuclease H (RNaseH), and integrase (IN) (1,61,63,122). A schematic representation of the structure of the HIV virion is illustrated in Figure 1.

1.3 The Viral Genome

All retroviruses contain a minimum of three genes, gag, pol, and env that encode the structural proteins as well as the enzymes necessary for viral replication. Lentiviruses, however, also contain additional genes, tat, rev, nef, vif, vpr, and vpu that are essential for efficient viral replication and persistence. The organization of each of the nine genes comprising the HIV genome is presented in diagrammatic form in Figure 2.

Transcription of the integrated provirus yields three species of mRNA that are differentially spliced: a 9.8 Kb full length transcript, 4.5 Kb singly spliced transcripts, and 2 Kb multiply spliced transcripts. The primary transcript is full length mRNA that is translated into a gag or gag-pol polyprotein. Gag (group specific antigen) is a 55 kDa precursor that is further proteolytically processed to yield the core proteins, capsid (p24), matrix (p17), and a nucleocapsid precursor (p15). The capsid protein forms the cone shaped shell beneath the matrix and is involved in viral assembly and disassembly. Structure-function studies have demonstrated that the carboxyl terminal domain is required for viral assembly, while the amino terminus of the protein is involved in infectivity. The matrix protein is N-myristoylated and performs an essential role in viral assembly by coordinating the intracellular transport and membrane association of the gag polyprotein. It is also responsible for recruitment of env to the plasma membrane. The nucleocapsid is further processed to yield p1, p2, p6, and p7 peptides. While little is known about the roles of p1 and p2, the p7 protein is multifunctional and assists in viral

Figure 1: **Structure of the HIV-1 Virion**

This schematic representation illustrates the approximate positions of Gag proteins, the Env glycoproteins, and the Pol-encoded enzymes integrase, reverse transcriptase, and protease (35).

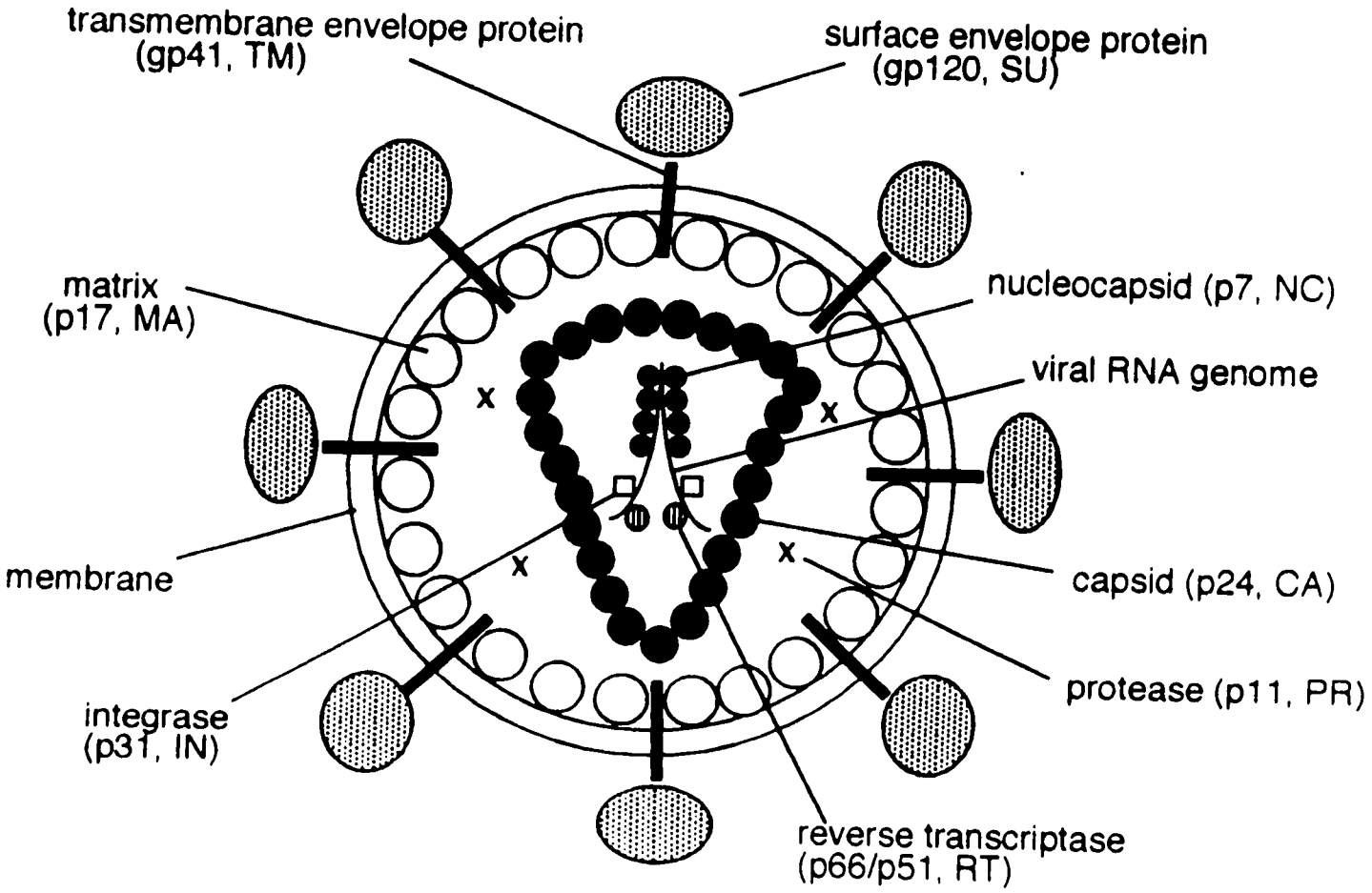
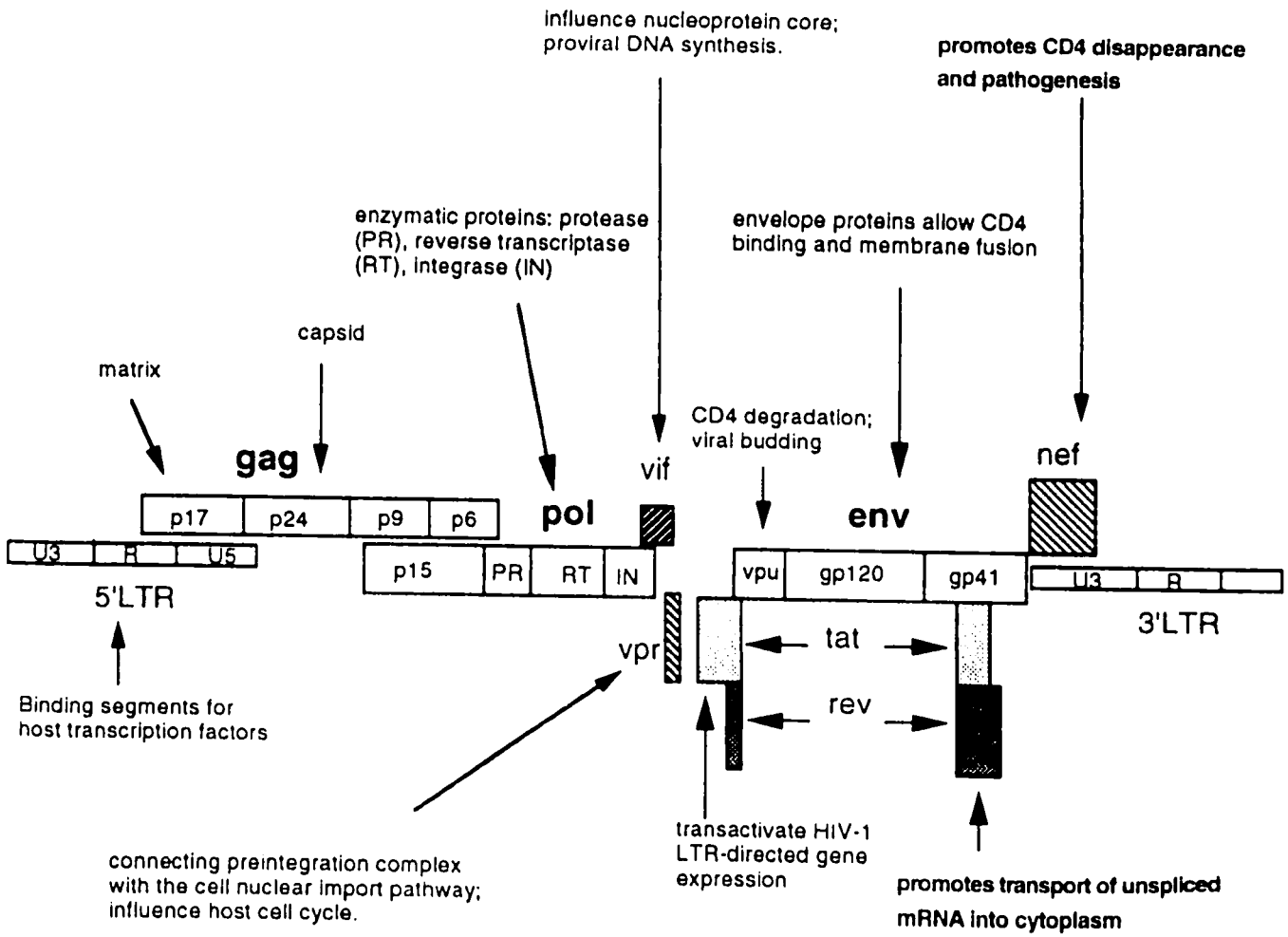

transmembrane envelope protein
(gp41, TM)
surface envelope protein
(gp120, SU)
matrix
(p17, MA)
nucleocapsid (p7, NC)
viral RNA genome
membrane
capsid (p24, CA)
protease (p11, PR)
integrase
(p31, IN)
reverse transcriptase
(p66/p51, RT)

Figure 2: **HIV-1 Genome Organization**

The HIV-1 genome is comprised of nine genes consisting of the structural genes, gag, pol and env, the accessory proteins, vif, vpr, nef, and vpu and the regulatory proteins tat, and rev. Descriptions of the known functions of each gene is included (63).


influence nucleoprotein core;
proviral DNA synthesis.
promotes CD4 disappearance
and pathogenesis
enzymatic proteins: protease
(PR), reverse transcriptase
(RT), integrase (IN)
envelope proteins allow CD4
binding and membrane fusion
capsid
matrix
CD4 degradation;
viral budding
gag
vif
nef
pol
env
p17
p24
p9
p6
U3
R
U5
p15
PR
RT
IN
vpu
gp120
gp41
U3
R
5'LTR
3'LTR
vpr
tat
rev
Binding segments for
host transcription factors
connecting preintegration complex
with the cell nuclear import pathway;
influence host cell cycle.
transactivate HIV-1
LTR-directed gene
expression
promotes transport of unspliced
mRNA into cytoplasm

packaging, and stimulates reverse transcription and RNA dimerization. P6 is involved in budding and assists with the packaging of vpr (35,63).

The pol (polymerase) open reading frame is translated exclusively as a gag-pol fusion protein by a translational frameshift mechanism in which the ribosome slips backward one nucleotide to allow the synthesis of gag-pol. Since the gag precursor products are required in greater proportion relative to the pol products, the gag to pol ratio in the mature virus is 20 to 1. The pol precursor is cleaved into the enzymes reverse transcriptase, integrase, and protease. Reverse transcriptase has three enzymatic functions. It is an RNA-dependent DNA polymerase , a DNA-dependent DNA polymerase, and a ribonuclease. All of these activities are essential for the synthesis of double stranded cDNA from viral RNA. Integrase incorporates the viral cDNA into the host genome via recognition of the 5' and 3' long terminal repeats flanking the HIV-1 genome and cleavage 2 to 3 base pairs from the 3' ends which are subsequently ligated to cellular DNA. Integration is permanent and semirandom with preference for highly bent DNA or transcriptionally active sites within the host genome (122). Protease is responsible for the processing of the gag, and gag-pol polyproteins which occurs at the surface of the host cell during budding.

The 4.5kb singly spliced mRNA encodes the proteins, env, vif, vpr, and vpu. The env gene encodes a 160 kDa precursor protein that is proteolytically processed to yield surface (gp120) and transmembrane proteins (gp41) which remain noncovalently linked as trimers on the surface of the virion. These proteins mediate docking to CD4 receptors and participate in viral entry by fusion to the plasma membrane of the target cell. Vif is a late gene product that enhances infectivity of progeny virions by interaction with host

cellular factors. Vpr is an accessory protein that prevents infected cells from replicating by causing them to arrest in the G_2 phase of the cell cycle. Since the viral LTR is more active in this phase than in other phases of the cell cycle, virus production is enhanced. Given that the half-life of infected cells is less than two days because of env-mediated cytopathicity, or immune cytotoxic responses, maximizing transcription by compromising cell division confers a selective advantage to the virus. Vpu is an integral membrane protein that functions in viral release and CD4 downregulation by binding CD4 in the endoplasmic reticulum and targeting it for degradation via the cytoplasmic ubiquitin-proteasome pathway (5,32,61,63,121,126).

The multiply spliced class of mRNA encodes the proteins, tat, rev, and nef. Tat is an essential transactivator protein that interacts with a stem-loop structure formed in the nascent RNA and increases the stability of the RNA polymerase as well as the frequency of RNA initiation. Rev (regulator of virion protein expression) is a nucleolar phosphoprotein that interacts with a cis-acting RNA loop known as the rev-response element (RRE) in the viral env RNA and mediates nucleocytoplasmic export of partially spliced and full-length mRNAs. Nef (negative factor) is a myristoylated cytoplasmic membrane protein that is expressed soon after infection. It is extolled to be an important negative regulator of HIV replication and is thought to play a role in the establishment of latent infection by interference with NF-KB mediated activation (5). It also exerts an indirect effect on viral gene expression by influencing cell physiology. Nef may stimulate cell proliferation, apoptosis or inhibit T cell activation. It also maintains high viral loads and is responsible for downregulation of CD4 receptors by accelerating endocytosis through clathrin-coated pits. Patients harboring a mutation within the nef gene display

normal CD4 levels with no sign of progression to AIDS (1,32,56,61,63,122,126).

1.4 The Viral Life Cycle

Virions adsorb to the CD4 receptor of the host cell via gp120 (1,56,61,118,122). A conformational shift from a nonfusogenic state leads to exposure of the domains on gp41 that are needed for pH-independent fusion with the plasma membrane (16,61). The virus is then uncoated by an ill-defined mechanism and its contents are liberated into the cytoplasm where reverse transcription occurs 4 to 6 hours post-infection (1,61,122). The reaction is initiated when a tRNA-lys3 primer anneals to the primer binding site (PBS) located 100 to 200 nucleotides from the 5' end of the viral template to produce an RNA-DNA hybrid. The RNA is degraded by the ribonuclease activity of reverse transcriptase, while the strand displacement activity allows the minus and plus strands of DNA to use each other as templates to complete their synthesis. Once viral RNA is converted to double stranded cDNA, it is transported to the nucleus as a preinititation complex that includes integrase, matrix, reverse transcriptase, and vpr. Nuclear localization is directed by vpr which lacks a nuclear localization signal but appears to function by connecting the preinitation complex with the nuclear import machinery of the host cell (122). Following proviral integration, RNA synthesis is initiated within the 5' long terminal repeat between the junction of the U3 and R regions (63). Initially, transcription is terminated prematurely due to abortive elongation. Tat enhances elongation by binding to the TAR (transactivating response elements) stem-loop formed on the nascent RNA transcript as a tat-cyclin T-cdk9 complex. Cyclin T binds tat and increases its affinity and alters its specificity for TAR RNA, while cdk9 phosphorylates RNA polymerase II and stimulates

processive elongation (122). Early mRNA transcription also relies on the interaction of cellular factors such as Sp1 and nuclear factor kB (NF-KB) with the HIV-1 promoter which can further enhance transcription levels (61). The HIV-1 genome is transcribed to produce three classes of differentially spliced mRNA which predominate at various stages of the life cycle. Early in infection, the multiply spliced transcripts are expressed at high levels and are exported from the nucleus first (56,61). Once rev is translated in the cytoplasm, it returns to the nucleus where it binds as an oligomer to the rev response elements (RRE) on the nascent full-length and singly spliced transcripts and recruits the cellular nuclear shuttling protein, exportin-1 and Ran-GTP. This complex is transported through the nuclear pore to the cytoplasm where hydrolysis of GTP to GDP results in disassembly of the complex and translation of the proteins gag, pol, and env. The env precursor, gp160 is synthesized in the endoplasmic reticulum and undergoes several posttranslational modifications. The protein forms a trimer, and is heavily glycosylated before being cleaved into the transmembrane protein, gp41 and the surface protein, gp120. This noncovalently linked complex is then translocated to the cell membrane for virus assembly. Since the CD4 and env proteins are both synthesized within the endoplasmic reticulum, premature binding of these two proteins can inhibit env transport or formation of a functional gp41-gp120 complex. As a result, the CD4 receptor is targeted for removal from the ER by the accessory factor, vpu which binds to CD4 and signals it for degradation via the ubiquitin-proteasome pathway (1,53,61,122). The cell surface expressed CD4 receptors are degraded by the endosomal pathway by binding to Nef. The gag-pol polyprotein is synthesized in ribosomes from unspliced mRNA (1,61,122). The genome assembles in the cytosol where the gag and gag-pol precursors

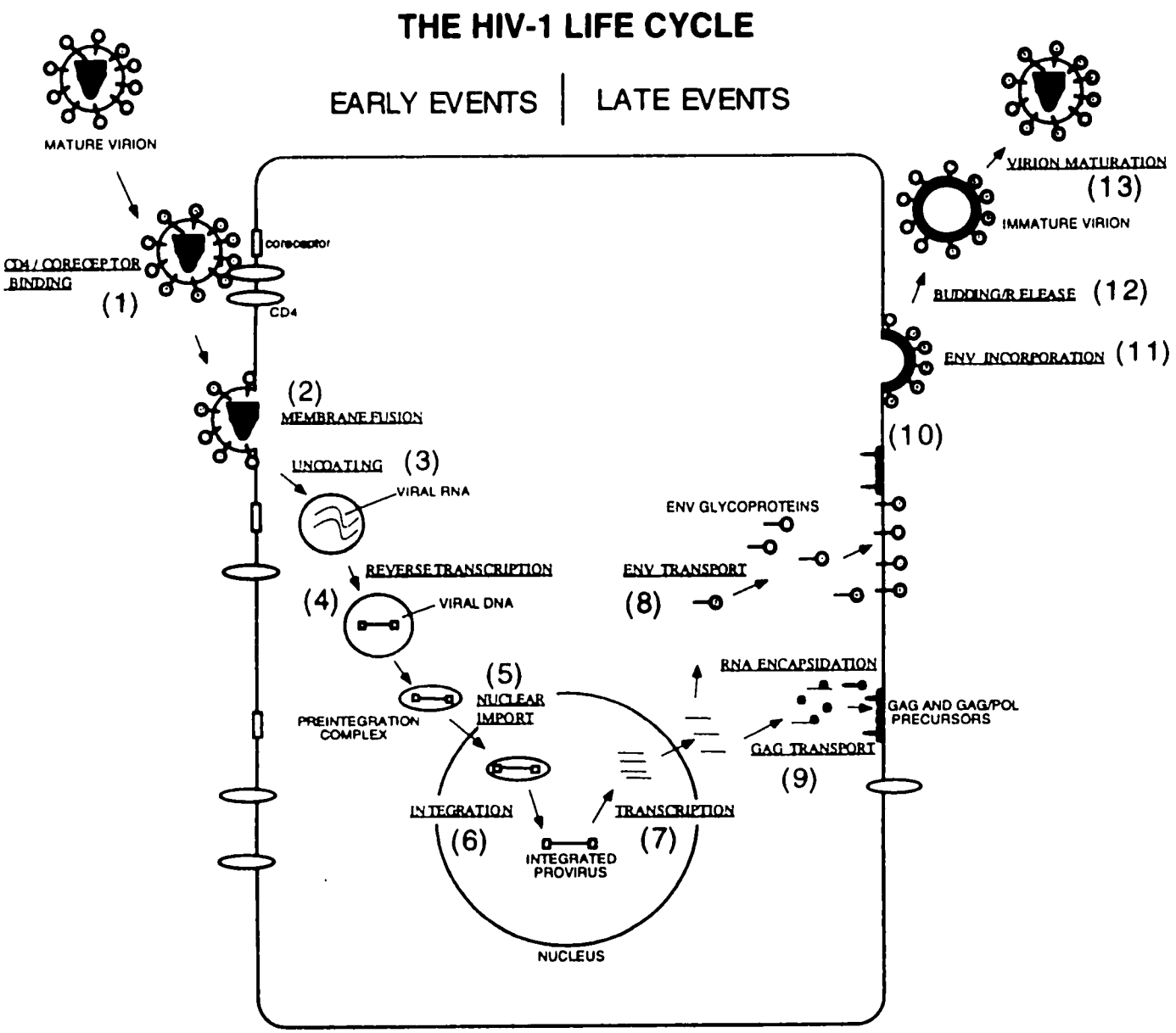
translocate to associate with the plasma membrane containing the env gp160. The polyproteins are cleaved shortly after budding by protease to yield the independent enzymes, reverse transcriptase, integrase and protease and the structural proteins, matrix, capsid, and nucleocapsid which rearrange during the maturation process to form an infectious virus (1,35,122). The complete process is illustrated in Figure 3.

1.5 HIV-1 and Host Factors

Throughout the viral life cycle, HIV-1 uses multiple strategies to exploit the host cell machinery in order to enhance its replication and pathogenesis. Virus entry is mediated by envelope protein interactions with cell surface CD4 receptors and the chemokine coreceptors, CCR5 and CXCR4 (101,119). This is followed by viral uncoating which is thought to be promoted by the interaction of capsid protein with cyclophilin A which was originally characterized for its specific binding to cyclosporin A (48). The preintegration complex (PIC) also appears to be a dynamic network of viral and cellular proteins that are important for conformational stability and efficient reverse transcription. At this stage, contacts are made with cell cytoskeletal proteins such as actin microfilaments that provide a scaffold along which the preintegration complex can translocate to more preferred sites in the cell. In addition, vif, vpr, rev, nef and reverse transcriptase have been reported to be modulated by phosphorylation via interactions with cellular kinases. A lack of proofreading activity in reverse transcriptase contributes to a high mutation rate and viral escape variants. The association of vpr with the DNA repair enzyme, uracil DNA glycosylase, however may restrict these mutations to those

Figure 3: **The HIV-1 life cycle**

Major steps in the virus replication process are illustrated. Early events include adsorption , membrane fusion, viral uncoating, reverse transcription, nuclear import, and integration. Late events are defined by transcription, Gag and Env transport to the cellular plasma membrane, followed by budding and virion maturation (35).


THE HIV-1 LIFE CYCLE
EARLY EVENTS
LATE EVENTS
MATURE VIRION
CD4 / CORECEPTOR BINDING
(1)
CORECEPTOR
CD4
(2)
MEMBRANE FUSION
UNCOATING
(3)
VIRAL RNA
REVERSE TRANSCRIPTION
(4)
VIRAL DNA
(5)
NUCLEAR IMPORT
PREINTEGRATION COMPLEX
INTEGRATION
(6)
INTEGRATED PROVIRUS
TRANSCRIPTION
(7)
NUCLEUS
ENV GLYCOPROTEINS
ENV TRANSPORT
(8)
RNA ENCAPSIDATION
GAG AND GAG/POL PRECURSORS
GAG TRANSPORT
(9)
(10)
ENV INCORPORATION
(11)
BUDDING/RELEASE
(12)
VIRION MATURATION
(13)
IMMATURE VIRION

that enhance fitness as opposed to those that could compromise viral survival (48). The preintegration complex then translocates to the nucleus by utilization of the host cell's nuclear import machinery. Successful integration into chromosomal DNA is influenced by the interaction of viral integrase with the host protein, Ini1 which is a known component of the chromatin remodeling complex, SWI/SNF. This association helps target the preinitiation (PIC) to transcriptionally active sites within the host chromosome. Other cellular proteins that can enhance integration efficiency include human high mobility group (HMG) and barrier-to-autointegration (BAF) protein which prevent PIC self-destruction by inhibiting autointegration. Once integrated, the provirus is subject to transcriptional control by multiple cellular factors such as NF-KB, Sp1, USF, YY-1, and LSF (reviewed in reference 48). In addition, the virus has evolved its own mechanism of enhancing transcription by encoding the potent transactivator, tat that cooperates with multiple transcription factors such as PTEFb, p300/CBP, TFIID, Sp1 and TIP30 (Tat interacting protein, 30kDa). Following transcription, the mRNA is processed by the host cell splicing machinery. Rev is a product of the multiply spliced transcripts that mediates nucleocytoplasmic export of unspliced and singly spliced transcripts by forming a multiprotein complex with the cellular proteins, Ran-GTP, exportin-1 and nucleophorin. Once the proteins have been translated, they are targeted to the plasma membrane for assembly and release. This is facilitated by N-myristoylation of gag polyproteins by cellular enzymes and potentially by the human double stranded RNA binding protein, staufen (reviewed in reference 119).

1.6 The Role of Tat

Tat is an early gene product translated from a multiply spliced transcript that is essential for optimal HIV-1 transcription and viral replication (5,49,50,56,98,122,125,128). Tat is a 15 kDa protein encoded by two exons. Exon 1 is coded by amino acids 1-72 and is analogous to the acidic amino terminal transactivation domain of classical transcription factors. This region includes a cysteine-rich sequence that chelates zinc in order to facilitate *in vitro* dimerization as well as a basic domain that is required for nuclear translocation and for interaction with TAR. The sequence of the basic region appears to be irrelevant for tat binding specificity, however the overall charge remains critical for maintaining a high degree of affinity for TAR (17,49,50,95,98,125). The second exon codes the carboxyl terminal domain of tat and includes an arginine-glycine-glutamate sequence involved in integrin binding to cell surface receptors (5,49,50,125).

In the presence of tat, viral gene expression is enhanced 10- to 1000-fold by increasing the proportion of transcripts that extend to the end of the 3' terminus. In the absence of tat, most transcripts terminate prematurely by abortive elongation and consequently the virus remains latent and nontoxic to the host cell (5,56,122).
The mechanism by which tat mediates transcriptional activation involves an interaction with specific cellular factors and the TAR element. TAR is positioned at the start of the 5' nascent transcript and has an extensive secondary structure that includes a stem, a loop, and a bulge. Tat is known to associate weakly with the UCU sequence (nucleotides +22 to +24) within the bulge region and in so doing recruit a complex of the host factors, cdk9 and cyclin-T collectively referred to as the positive transcription elongation factor

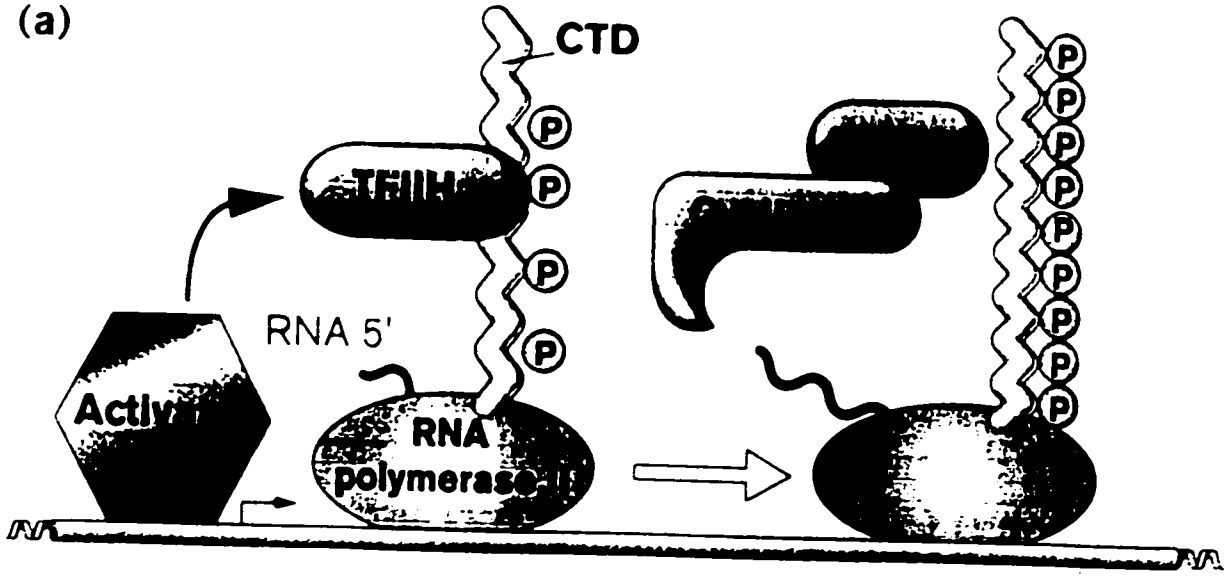
(P-TEFb) (95). The interaction of tat with P-TEFb alters the conformation of the tat protein to mediate more favorable binding to the TAR loop. Several lines of evidence have also demonstrated that tat can associate with the initiation factor, TFIIH which partially phosphorylates the tandem heptapeptide repeat (YSPTSPS) within the carboxyl terminal domain of RNA polymerase II. This is necessary for promoter clearance and elongation. Once the RNA is 30 to 50bp long, tat/TFIIH is exchanged for tat/PTEFb which further enhances phosphorylation and launches a highly processive RNA polymerase II (95,128). The mechanism of tat-mediated transactivation is displayed in Figure 4. Whether tat remains at the promoter or travels as a passenger with RNA polymerase has been a controversial issue. Based on biochemical evidence, some tat protein may be stably associated with the promoter by contacting the DNA-bound enhancer, Sp1 in a synergistic and specific manner (5,17,50). Thus, there is evidence that tat exerts its transactivation function by increasing the rate of initiation, by stabilizing transcription elongation or by a combination of these two processes (45,93).

A rather unusual property of tat protein is that it is able to readily cross the plasma membrane into adjacent cells by endocytosis while still retaining its ability to localize to the nucleus and transactivate numerous target genes. Such transcellular transactivation would enhance HIV-1 pathogenicity by activating latent proviral reservoirs (5,124). This property of tat is being exploited in attempts to establish novel antiretroviral therapy. Anti-tat antibodies or specific compounds that are capable of interacting with extracellular tat and sequestering it may be useful in preventing its entry into cells and reducing HIV-1 infectivity. There is mounting evidence from tissue culture experiments that the addition of anti-tat antibodies can halt the progression of viral infection and

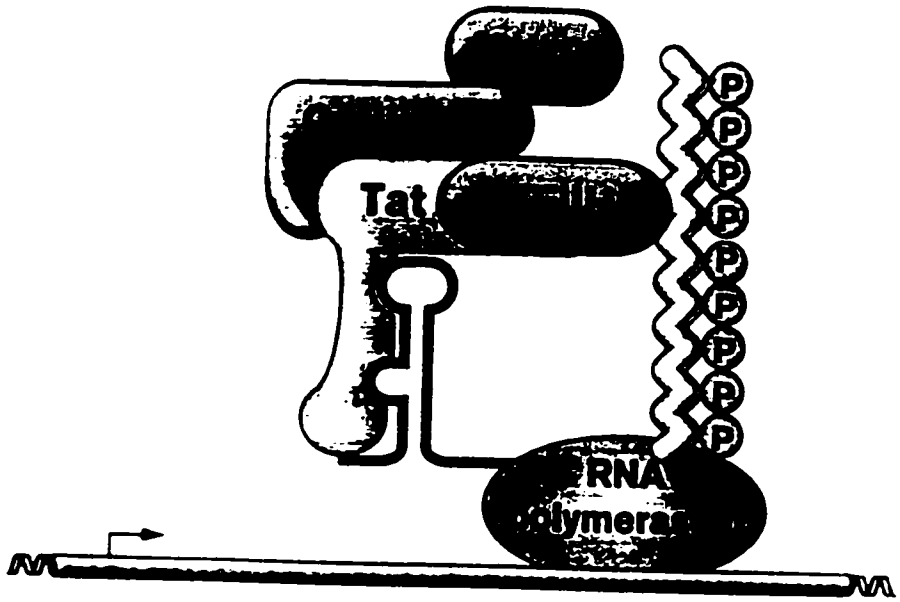
Figure 4: The mechanism of tat transactivation

A two stage model for phosphorylation of the RNA polymerase carboxyl terminal domain is illustrated. TFIIH initiates partial phosphorylation. Recruitment of PTEFb and tat enhances phosphorylation and launches a highly processive elongation complex (128).

(a)



(b)



patients who develop tat antibodies show increased life spans (5). Alternatively, one group of researchers coupled tat to a modified caspase-3 protein containing the gag cleavage site thus enabling only the HIV-1 infected cells susceptible to programmed cell death while simultaneously leaving the uninfected cells of the immune system intact (125).

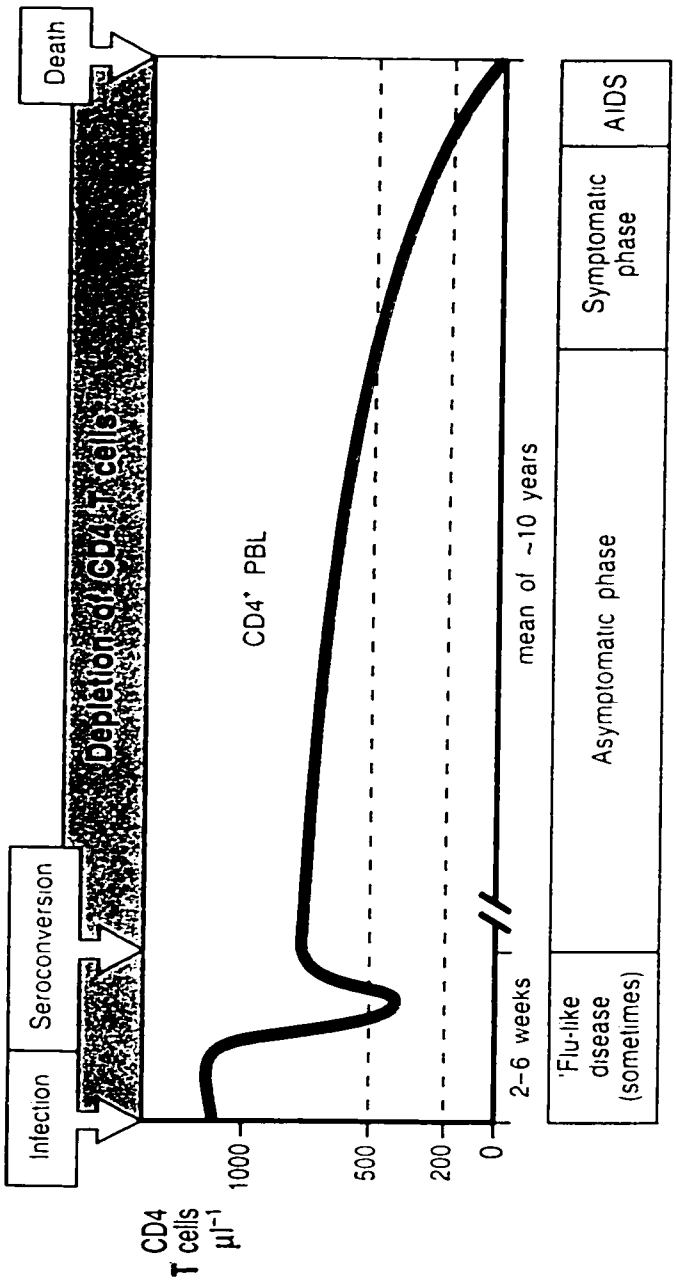
1.7 The Effect of HIV-1 on the Immune System

HIV-1 infects CD4+ T-lymphocytes and macrophages and is present as free virions in the blood stream, and body fluids. Four phases define a typical course of infection. These stages are reviewed in diagrammatic form in Figure 5. The first phase has a duration of 4 to 6 weeks and is characterized by flu-like symptoms and a sharp decline in CD4-expressing cells. The acute viremia is temporarily controlled by activation of CD8+ cytotoxic T-lymphocytes (CTL) as well as subsequent seroconversion in which antibodies are generated against viral peptides. This immune response helps restore CD4+ T cells, but it fails to completely eradicate the virus. This phase is followed by a long asymptomatic period in which the virus continues to replicate and CD4+ cells are gradually depleted (1,2,51). The loss of these T cells results in heightened susceptibility to malignancies and opportunistic pathogens including parasites, bacteria, fungi, and other viruses (32). AIDS is defined by detectable levels of virus and CD4 counts that have fallen below 200 cells/uL of blood. At this phase, the expected life span is less than one year (1,2).

The innate, humoral, and cellular immune systems co-operate to control most

Figure 5: **Typical course of HIV-1 infection**

This is a graphical illustration of the time line followed by HIV-1 infection with respect to CD4+ T-cell levels. The first few weeks are characterized by mild illness, high viral titers and a dramatic depletion in CD4+ T-cells. The ensuing adaptive immune response is able to control viremia and partially restore CD4 levels which gradually continue to decline. As the immune system is increasingly compromised, opportunistic infections occur and eventually AIDS develops (2).


Death
Infection
Seroconversion
Depletion of CD4+ cells
CD4+ PBL
CD4 T cells µl-1
1000
500
200
0
2–6 weeks
mean of ~10 years
'Flu-like disease (sometimes)
Asymptomatic phase
Symptomatic phase
AIDS

viral infections. Despite a vigorous immune response, HIV has evolved numerous strategies to ensure a lifelong and persistent infection.

The hallmark of AIDS is depletion of CD4-expressing T-lymphocytes and ironically, their activation creates ideal conditions that support infection, integration and dissemination of HIV. The T cell receptor on the CD4+ cell recognizes viral peptides presented on the class II major histocompatibility complex (MHC) on the antigen-presenting cell. This interaction facilitates activation and clonal expansion of the CD4+ cell which is then competent to secrete cytokines such as interleukin-2 and interferon-γ to stimulate CTL or interleukin-4, -5, -6, and -10 to induce a B-cell response (2,39,51). Thus, these cells play a pivotal role in coordinating an impressive immune response and their depletion results in severe impairment in the ability to control the infection.

When HIV infects a target cell, a sequence of events is orchestrated that culminate in the display of viral peptides in conjunction with the class I MHC on the cell surface. The T cell receptor on the CTL recognizes the viral antigen and triggers direct lysis of the infected cell (1,39,51,72). Alternatively, it can secrete antiviral cytokines such as interferon-γ and tumor necrosis factor-α or β-chemokines such as MIP1α, MIP1β, or RANTES which function by binding HIV coreceptors on the surface of the target cell thus preventing entry (39). This cell-mediated immune response occurs early in infection and is believed to be responsible for resolving the viral burden during the acute phase (39,51,82). However, there is mounting evidence that it also exerts a strong selective pressure on the production of viral escape variants (39,51,53,72). Mutations arise due to rapid viral replication, a lack of proofreading activity within the reverse transcriptase, and its intrinsic ability to jump between templates during cDNA synthesis that results in a

high frequency of recombination events (1,51,126). The error rate of reverse transcriptase was estimated to be as high as 10^{-4} (51,122). This predicts that there will be one error per viral genome per replication cycle and since billions of cells are infected daily, every conceivable mutation can be generated thousands of times over in a single infected individual each day (51).

Immune detection can also be evaded by downregulation of MHC proteins expressed on the surface of the infected cell. This is accomplished by three distinct mechanisms and three viral accessory proteins. CD4+ T-cells expressing high levels of Nef are protected from MHC-restricted CTL destruction. The mechanism involves a Nef-mediated conformational change in the cytoplasmic domain of the MHC which stimulates internalization of MHC followed by degradation via the endosomal pathway (53). In addition, Nef redirects MHC molecules to clathrin-coated vesicles in the golgi apparatus. MHC-I downregulation by Nef is selective for the HLA-A and –B classes, but not HLA-C that is involved in inhibition of cytolysis mediated by natural killer (NK) cells. Thus, the virus has evolved a highly proficient method of minimizing antigen presentation to CTL while simultaneously avoiding NK-mediated cytolysis (51,53,72). Tat-mediated MHC-I downregulation occurs at the transcriptional level when Tat binds to the TAFII250 component of TFIID and prevents it from interacting with the MHC-I promoter. In an alternative model, tat represses the β_2-microglobulin promoter by binding to TAR (53).

Recently, it was reported that vpu may also function to reduce surface expression of MHC-I by degrading newly synthesized molecules potentially by a mechanism analogous to CD4 downregulation (53).

Within one to three months of acute infection, antibodies are produced that are directed first against the gag proteins, p24 and p17 followed by env, and pol. The source of these proteins is likely to represent viral debris from lysed cells. The antibodies that are produced against the envelope proteins recognize the V3 hypervariable loop of the gp120 subunit, and the neutralization-face (the side of gp120 that is not hidden when env oligomerizes). The efficacy of antibody-mediated immunity is compromised because of antigenic drift, epitope masking by envelope glycosylation, overlapping variable loops that effectively shield more conserved elements in the complex, transient exposure, and occlusion of epitopes by oligomerization (39,51).

Other strategies employed by the virus to evade the immune system includes infection of immune privileged cells, induction of apoptosis of CTL by expression of Fas ligand, and the maintenance of a permanent viral reservoir (2,51,72).

1.8 Latency

The HIV-infected individual possesses several distinct cell populations. These include cells carrying actively replicating virus, cells infected with defective proviral genomes, cells that possess a transcriptionally silent, yet inducible integrated provirus, and a population that proliferates, and differentiates to compensate for losses in the former cells (71). Latent infection predominantly arises in resting memory CD4+ T-cells in which the virus is stably, and indefinitely maintained in accordance with the biological function of these cells. This pool of cells represents approximately 1% of the total population that includes both integrated and unintegrated forms of HIV (2,19,51,71).

Since most CD4+ T cells are quiescent *in vivo*, the virus will temporarily remain in a labile state of unintegrated latency for a period of hours to days when it is then degraded. While cells that undergo at least one round of replication prior to quiescence will maintain an integrated latent provirus that retains the ability to replicate upon antigen driven T-cell activation (71). Thus, latent infection plays an important role in the maintenance of a permanent reservoir to ensure long-lasting viral persistence (51). Furthermore, there is evidence that these viral reservoirs not only maintain the original wildtype form of the virus, but also the collection of quasi-species that evolved from the selective pressure imposed by therapy (10).

Latent infections are sustained by a mechanism that involves dynamic interactions of host cell factors with proviral DNA and proteins. This includes, but is not limited to "blocked early-stage latency" whereby there is an accumulation of multiply-spliced transcripts due to the absence of rev, a lack of NF-KB-facilitated activation of the HIV-1 LTR, a deficiency in one or several essential viral proteins such as tat, or chromatin architecture (10,14,71).

Given that the half-life of this reservoir is 44 months, eradication would require 60 years of intensive suppressive therapy (10,14). Thus, latently infected cells pose a serious clinical impediment because they are not targeted by conventional drug therapy and have the potential to reinstate viremia when treatment is discontinued (10). Furthermore, disease progression has been linked to a shift in the relative proportion of cells that possess a latent provirus to those that are actively replicating (71). Unless a mechanism can be elucidated that will permit clearance of this viral reservoir, it is likely that lifelong control will require lifelong treatment.

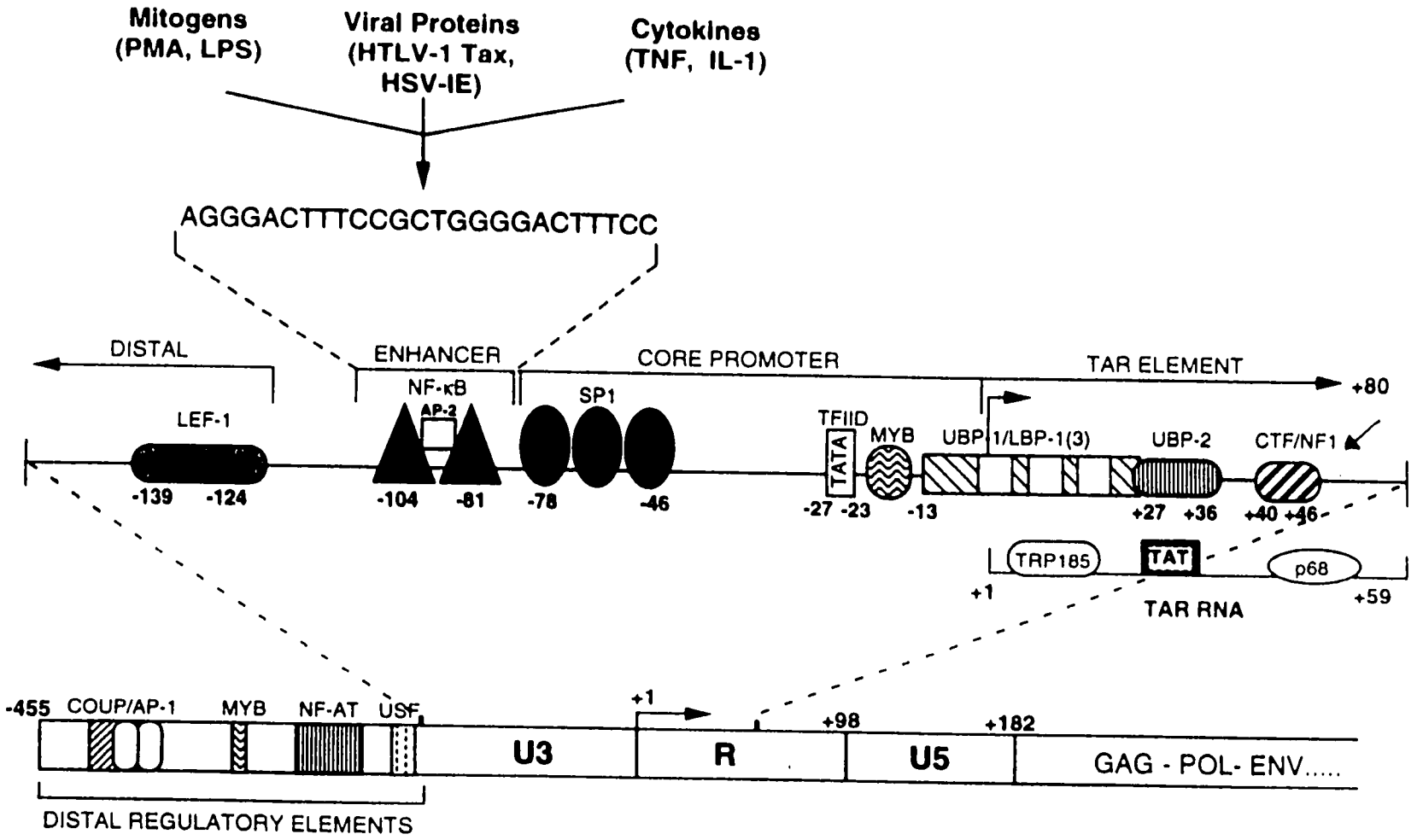
1.9 The Long Terminal Repeat

The long terminal repeat (LTR) is defined by the U3, R, and U5 regions which possess both upstream and downstream transcriptional regulatory elements (20,40,48,96,100,129). The start site is located at the junction of the U3 and R regions such that upstream control elements are found in U3 while downstream elements are located within the R and U5 regions (56). The HIV-1 LTR is divided into several discrete functional domains: a negative regulatory element (-340 to -184), an enhancer (-105 to -79), a basal promoter (-78 to -1), TAR (tat activation response element, +15 to +45) and the inducer of short transcripts (IST). While many of these control elements are common to most genes, TAR and IST are unique cis-acting elements of HIV-1. The IST is a DNA element that overlaps with TAR and functions as an enhancer to promote the synthesis of abortive transcripts. TAR is an RNA element present in both the viral genome and the mRNA that functions as an enhancer for the synthesis of processive transcripts (40,87,100). The organization of the long terminal repeat is shown schematically in Figure 6.

The NRE is responsible for the negative control of transcription and contains consensus binding sites for the transcription factors NF-AT1, ILF-1 (-216 to -254), USF (-159 to -173), GRE (-235 to-276), COUP, myb, GATA3, RAR (-300 to -350) and LEF-1 (-121 to -150). The mechanism by which viral expression is downregulated by this region is unknown (56,100,118). Mutational analysis of HIV-1 proviruses has further confirmed the presence of this element since deletion of this region resulted in enhanced virus

Figure 6: Structure of the HIV-1 Long Terminal Repeat

This is a schematic diagram of the HIV-1 5' promoter showing the U3, R and U5 regions which are further subdivided into the TAR DNA element, the core promoter, the enhancer, and negative regulatory element. The relative positions of cellular binding proteins are also designated (100).


Mitogens
(PMA, LPS)
Viral Proteins
(HTLV-1 Tax,
HSV-IE)
Cytokines
(TNF, IL-1)
AGGGACTTTCCGCTGGGGACTTTCC
DISTAL
ENHANCER
CORE PROMOTER
TAR ELEMENT
+80
NF-κB
AP-2
SP1
LEF-1
TFIID
TATA
MYB
UBP 1/LBP-1(3)
UBP-2
CTF/NF1
-139 -124
-104 -81
-78 -46
-27 -23
-13
+27 +36
+40 +46
TRP185
TAT
p68
+1
+59
TAR RNA
-455 COUP/AP-1
MYB
NF-AT
USF
+1
+98
+182
U3
R
U5
GAG - POL- ENV.....
DISTAL REGULATORY ELEMENTS

replication (5).

The enhancer region contains two 10-bp conserved binding sites (-109 to –79) for the NF-KB/rel B family of transcription factors (118). The nuclear levels of NF-KB are strongly correlated to the activation status of the T cell. In resting cells, NF-KB preexists in the cytoplasm associated with its inhibitor, IkB. In response to cytokine, mitogen, or viral protein stimulatory signals, IkB is phosphorylated causing it to dissociate from NF-KB. This exposes NF-KB's nuclear localization signal which facilitates its translocation. Once in the nucleus, NF-KB dimers bind to DNA where activation of transcription can occur. In this manner, the LTR NF-KB binding sites would permit a transcriptionally silent integrated provirus to be activated (5,100).

The basal promoter is a GC-rich region that possesses three tandem Sp1 boxes (17,84). In addition a fourth Sp1 site was identified at –433 to –441 nucleotides relative to the start site of transcription that is involved in negative regulation of the HIV-1 LTR (84). Sp1 contains three carboxyl terminal domain zinc-finger motifs involved in DNA binding and two glutamine-rich transcription activation domains. There is evidence that tat and Sp1 can form a protein-protein complex that alters the phosphorylation state of Sp1 via recruitment of the enzyme DNA-PK. The hyperphosphorylated status of Sp1 then facilitates interaction with members of the basal transcription complex including TBP, TAF110, and RNA polymerase II to stimulate transcription from the HIV-1 LTR (17). Mutation or deletion of the Sp1 sites results in significant loss of basal transcription and compromises the ability of tat to transactivate the HIV-1 LTR (93). In contrast, deletion of these sites within an infectious molecular clone of HIV-1 resulted in the production of replication-competent virus and the detection of upregulated levels of other

transcriptional activators such as NF-KB. Indeed, this HIV-1 mutant failed to replicate in T-cells lacking NF-KB which suggests that the function of Sp1 can be partially replaced by other factors (5). Vpr was also demonstrated to associate with Sp1 and transactivate the HIV-1 promoter prior to activation by NF-KB (118).

The core promoter extends from -30 to +50 and includes both an initiator element and a TATA box flanked by E boxes homologous to the consensus sequence CANNTG (5,80,118). The TATA element extends from nucleotides –27 to –23, while the direct repeat E box motifs are positioned between –38 to –33 and –21 to –16 relative to the start site of transcription. These elements are recognition sites for the basic-Helix-Loop-Helix/Leucine Zipper class of transcription factors, while the TATA box recruits the transcription preinitiation complex which includes both general and specific host cell transcription initiation factors and RNA polymerase II. Mutational studies have verified the significance of these elements in the regulation of basal and tat-induced gene expression from the HIV-1 LTR. Mutation of the TATA box but not the E boxes compromised TBP and TFIID binding, while mutations in the E-box motifs reduced tat-mediated transactivation and the binding of bHLH class of transcription factors. Thus, the interaction of positive and negative factors with the sequences flanking the TATA box are a critical determinant in modulation of HIV-1 gene expression either by cooperation with or exclusion of members of the basal preinitiation complex (80).

The initiator element is a pyrimidine-rich region whose core maps to -2 to +7 of the HIV-1-LTR and is known to interact with the initiation factor, TFII-I, as well as the transcription factors USF, and YY-1. A second initiator element has been mapped to nucleotides +35 to +60 and bears considerable sequence similarities to the first initiator

(28). It is therefore conceivable that an additional preinitiation complex, distinct from the TATA box, assembles at this region and enhances transcription. USF is known to be stimulatory which is mediated by a mechanism that involves interaction with TFII-I. YY-1 is a nuclear matrix protein that was demonstrated to cooperate with another factor, LSF and inhibit HIV-1 expression and virus replication. There is a low affinity binding site for LSF that extends between -38 and -16 overlapping the TATA box, and high affinity sites spanning the -4 to +21 region of the LTR. *In vitro* transcription studies demonstrated that LSF could prevent binding of TFIID to the TATA box as well as block elongation of RNA polymerase II if the preinitiation complex was assembled prior to the addition of LSF (81,129). The initiator element also possesses a putative binding site for CTF/NF1 (CAAT-box transcription Factor/Nuclear Factor-1), but a role for this protein in HIV-1 transcription is yet to be defined (5,118).

1.10 HIV-1 Transcription

Gene expression is modulated by numerous cis-acting DNA elements present in the HIV-1 long terminal repeat (20,40,48,96,100,129). These elements are binding sites for general and sequence-specific transcription factors that activate or repress transcription from a target gene. While RNA polymerase II is composed of 12 different subunits, it cannot transcribe DNA without the participation of host cellular factors. A step-wise assembly model for the formation of a pre-initiation complex at the core promoter has been proposed. An initial committed complex is formed by the binding of the initiation factor, TFIID to the TATA box within the DNA minor groove. TFIID is

composed of TATA-binding protein (TBP) and TBP associated factors (TAFs) whose role it is to support activation from gene specific transcription factors. An additional factor, TFIIA is required *in vivo* to counteract repressor proteins that thwart TFIID binding to the TATA box by direct interaction with TBP. TFIID can further recruit additional class II general factors such as TFIIB followed by RNA polymerase and TFIIF. TFIIF can complex to the polymerase in the absence of DNA or other factors and functions by directing it to the promoter and away from non-specific DNA sequences (12,73,80,94). The RNA polymerase exists in two states. The hypophosphorylated form IIA has a greater binding affinity than the hyperphosphorylated form IIO and is preferentially recruited to the preinitiation complex. The subsequent association of TFIIE and TFIIH completes the basal transcription complex. TFIIH is a multi-subunit protein with several biological functions. The kinase activity phosphorylates the RNA polymerase carboxyl terminal domain, while the helicase and ATPase functions activate the initiation complex and melt the DNA surrounding the transcription start site to facilitate promoter clearance (12,80). The elongation complex consists of TFIIF and the RNA polymerase. At this stage, the other general transcription factors dissociate and are recycled to facilitate a second round of initiation (12,73,80,94).

1.11 Chromatin Structure and its Effects on Transcription

Gene expression is directly correlated to chromatin architecture. DNA combines with the histones, H2A, H2B, H3, and H4 to form octameric structures known as nucleosomes whose precise organization represents the first order of chromatin folding

(70). Histones are highly conserved, basic proteins containing an abundance of lysine and arginine residues whose positive charges facilitate association to the negatively charged sugar-phosphate backbone of DNA. The nucleosome core is wrapped with 146bp of DNA with 54bp of linker DNA between each core particle. Histone H1 is located at the point of DNA entry and exit outside the nucleosome and is necessary for higher order chromatin folding. The transition between various forms of chromatin is associated with reversible covalent modification of the histones. The enzyme, histone acetyltransferase (HAT), can acetylate the lysine residues within the N-terminal domains of the core histones. Several cellular proteins have been demonstrated to possess HAT activity including TAFII250, hGCN5, P/CAF, p300/CBP, SRC1, and ACTR (reviewed in reference 70). These enzymes cause the chromatin to decondense by reduction of H1 binding to the nucleosome core and neutralization of the positive charges. Chromatin decondensation facilitates transcriptional activation by a mechanism that involves repositioning of the nucleosomes so that they no longer obscure the promoter region or binding sites for activators (70). In contrast, transcriptional repression is correlated to highly condensed chromatin and recruitment of the enzymes, histone deacetylase 1 and 2 (HDAC). In this model, DNA binding repressors interact with adapter proteins such as mSin3A, B and/or N-CoR/SMRT that link the sequence-specific transcription factor with the deacetylase (8,83).

The HIV-1 provirus is incorporated into the host chromosome with two precisely positioned nucleosomes termed Nuc-0 and Nuc-1. Nuc-0 is located upstream of nucleotide −164, while Nuc-1 is positioned at the start site of transcription and consequently may contribute to proviral latency. Often, nucleosomes are organized at

regularly spaced intervals, however the HIV-1 LTR appears to be an exception. While the region located between Nuc-0 and Nuc-1 is large enough to accommodate an additional nucleosome, it never does so. Instead, it is bound by transcription factors such as Sp1, USF, TBP and LEF-1. Likely, the interaction of these factors with their cis-acting DNA elements induces bending that renders this region unfavorable to nucleosome assembly. These observations implicate a pivotal role for histone acetylation in the activation of the HIV-1 LTR. The mechanism involves tat recruitment of the histone acetyltransferase, p300/CBP to the promoter during transcription initiation (70).

1.12 Therapeutic Strategies

Despite an intensive effort, an effective therapy against AIDS has not been achieved. While current treatment strategies are capable of limiting viral replication and improving immune competence, their long term use is unrealistic. A complicated drug regimen, metabolic side effects, and high costs make adherence difficult and the inevitable emergence of quasi-species resistant to therapy only compound the problem (19,118,122). 15 drugs currently constitute the anti-AIDS arsenal belonging to three classes: the nucleoside analog reverse transcriptase inhibitors (NRTI), the nonnucleoside reverse transcriptase inhibitors (NNRTI) and the protease inhibitors (PI). Highly active antiretroviral therapy (HAART) involves the administration of these drugs in various combinations to control the infection. Since many of these drugs have similar mechanisms of action and chemical structures, there are emerging issues related to cross-resistance. Consequently, there is an aggressive search for novel therapeutic strategies

that target the accessory proteins or critical steps in the viral life cycle. For example, diketo acids were identified as bona fide integrase inhibitors that function by preventing strand transfer. The HIV-1 entry process is also a potential target. In principle, inhibiting gp120-CD4 interactions, gp120-coreceptor binding, or gp41 function can prevent this process. Alternatively, since gag and vpu both play essential roles in the assembly and release processes, identifying compounds that can interfere with their function could prove to have important antiviral efficacy (19).

Immunotherapy strives to boost the weakened immune system in order to combat infection (39,82). This would include the use of cytokines such as interleukin-2 which functions by activating latently infected cells, thereby allowing antiretrovirals to have an effect (39,101). In supervised treatment interruptions, therapy is withheld in order to prevent reduction of the CTL response while simultaneously allowing only a limited amount of viral replication (39,101). In practice, any discontinuation of therapy was met with a rebound in viral titer, although improvements were seen in the form of reduced viral diversity (101). Initiation of therapy early in the acute phase was beneficial in the preservation of CTL responsiveness which are absent if therapy is delayed (101). In general, however, issues related to toxicity, sustainability of the response, and quality of life need to be considered before immunotherapy becomes a standard of care.

The development of an effective vaccine is the primary objective. The ideal properties of this vaccine should include a minimum of side effects, confer long-lasting efficacy, prevent autoimmunity, target latently infected cells and most importantly circumvent immune evasion strategies by inducing cell-mediated and humoral responses, and elicit neutralizing antibodies that react with all HIV strains and subtypes (61). Unfortunately,

efforts towards vaccine development have been met with several obstacles. The first is the lack of an exact animal model that would permit extrapolation of the results of vaccination trials. The current model system is based on Asian macaques which are susceptible to infection with the HIV-related virus, simian immunodeficiency virus (SIV). However, SIV cannot cause disease in African macaques, and therefore this species variability should serve as a caveat when interpreting results of vaccination trials in terms of human relevance (2). A further challenge is that any vaccine developed must combine both immunogenicity, and safety (76). An additional problem relates to the type of immunity that would be required to successfully contain the infection. The immune system mounts a vigorous response, and yet is unable to control the infection. There is, however, a population of people termed long-term nonprogressors that have been exposed to HIV, but fail to develop the disease. Understanding why these individuals manage to control their infection will be paramount in vaccine development (2,82). There is also great concern that an AIDS vaccine would attenuate acute infection, but not eliminate it. In addition, a vaccine based on a particular HIV-1 clade would not be able to protect against infection with viruses belonging to other clades (76). Finally, there are ethical issues that must be confronted once a vaccine has been developed. It would be immoral to conduct a trial in a high-risk population without employing other strategies to minimize exposure, although this may skew the results. Furthermore, some individuals would need to be given a placebo, rather than an active form of the vaccine. Obviously this would not be just since all participants deserve an equal opportunity to receive a potential treatment.

2. Transcription Factors

2.1 USF

Human upstream stimulatory factor is a highly conserved member of the basic-helix-loop-helix (bHLH) class of regulatory proteins that recognizes specific consensus sequences termed E box motifs (22,28,46,54). USF is constitutively and ubiquitously expressed in diverse cell types (46,115). In mammals, there are two USF genes that encode 44 kDa and 43 kDa proteins. The carboxyl terminal domain of these polypeptides contains similar bHLH motifs and consequently demonstrates similar DNA binding and dimerization specificities. The USF-specific region (USR) is a small domain found exclusively in USF proteins. It is located upstream of the basic region and is required for transactivation of promoters containing both TATA, and initiator elements. The amino terminus of these two proteins is more variable and comprises the transactivation domain. In most tissues, USF is found frequently as a heterodimer of the 43 and 44 kDa forms. While homodimers have been known to occur, they are considerably less abundant (91).

USF was initially identified as a DNA binding factor that could activate the adenovirus major late promoter (AD-ML) *in vitro* (28). It was also demonstrated to negatively regulate transcription of the HIV-1 promoter when it binds to an E box element positioned at –173 to –157 (22). In contrast, when USF binds to an E box motif located near the start site (-5 to +9), it stimulates transcription and induces DNA bending that likely facilitates interactions with other co-activators (22). The effect of USF on the HIV-1 LTR appears to be cell-type specific since it was demonstrated to function as a transcriptional activator in T-cell lines, and a repressor in epithelial cells (75).

Furthermore, USF can recognize initiator elements within the HIV-1 LTR located at (Inr 1) –5 to +9 and (Inr 2) +29 to +42 and activate transcription in a concentration-dependent manner (28). This result implies that USF can function through two distinct pathways. In the absence of an intact E-box, it can stimulate transcription through initiator elements and when Inr 1 and Inr 2 are absent, it exerts its stimulatory function through E box motifs (22,28). However, USF exhibits a greater binding affinity for E box elements than initiator sequences. The mechanism for USF-mediated stimulation through initiator elements is still uncertain and may involve direct binding, interactions with other transcription factors, or even an indirect effect. There is mounting evidence which suggests that USF interacts with additional cellular factors that likely belong to the bHLH family (28). One probable candidate is the immunologically related, initiator binding protein, TFII-I (102). TFII-I can bind to both initiator and E box elements independently and synergistically with USF to positively modulate transcription (28). In contrast, when TFII-I complexes with another bHLH protein, c-Myc, it represses transcription (60).

Furthermore, since the Inr 1 site overlaps binding elements for the cellular factors YY-1, and LSF and the Inr 2 sequence overlaps the TAR element, it raises the prospect that the HIV-1 core promoter is modulated by a dynamic network of positive and negative factors (28).

2.2 LSF

Leader binding protein-1 (LBP-1) is a ubiquitously expressed cellular factor that is analogous to Late SV40 Factor (LSF) and the murine homologue, αCP2 (129). There

are multiple proteins in the LSF family produced by alternative splicing mechanisms from two distinct genes (20,129). The carboxyl terminal domain contains proline, glutamine, and serine/threonine-rich sequences typical of classical activators, whereas the amino terminus is considerably more highly conserved and appears to function in DNA binding. LSF recognizes derivatives of the motif 5'-TCTGG-3' (67). It was demonstrated to bind to its recognition site as dimers and cooperate with other factors to positively or negatively regulate transcription depending on the promoter context (20,96,117,129). LSF activates the SV40 and adenovirus promoters by increasing the rate of association of TFIIB to the preinitiation complex. Given that TFIIB does not appear to remain at the promoter once the RNA polymerase has cleared and is not a member of the elongation complex, it is likely that LSF could re-recruit TFIIB to stimulate reinitiation (117).

The HIV-1 promoter possesses three high affinity LSF binding sites overlapping the IST, TAR and initiator elements at nucleotides –4 to +1, -7 to +11, and +17 to +22 as well as a lower affinity site overlapping the Sp1 and TATA boxes at –38 to –16 (67,81). While LSF was shown to directly repress transcription from the LTR *in vitro*, the effect was not observed in transient transfections (129). On the basis of the *in vitro* studies, it was determined that repression occurred at the levels of initiation and elongation. LSF could block TFIID binding to the TATA box, and thus prevent assembly of the preinitiation complex. However, prebinding of TFIID followed by the addition of LSF permitted initiation, but blocked elongation by stimulating RNA polymerase pausing. The precise mechanism involves LSF binding to its low affinity site and outcompeting elongation factors that mediate promoter clearance. Furthermore, LSF may restrict elongation of HIV-1 transcripts by cooperating with the IST element in the synthesis of

short abortive transcripts in the absence of tat. The addition of tat, however, was able to reverse LSF-mediated repression and remove the elongation restriction imposed on RNA polymerase by an indeterminate mechanism. Altogether, understanding the exact role(s) of the LSF recognition sites on the HIV-1 promoter has been complicated by their multiplicity and dispersion among the TATA, Sp1, IST, TAR, and initiator elements (81).

2.3 YY-1

Yin Yang-1 was the first factor described to mediate transcription through initiator elements and was originally cloned because it associated to an E1A site on the adenoassociated virus (AAV) P5 promoter (6,111). The carboxyl terminal domain contains four zinc finger motifs belonging to the GLI-Kruppel family, all of which are absolutely required for sequence-specific DNA binding (6,13,113). The amino terminus contains a bipartite acidic transactivation domain followed by a histidine-rich region whose function is yet undetermined (13,113). The central region is defined by residues 201-333 and enables YY-1 to associate with numerous proteins including, but in no way limited to c-myc (7,78,112,114,131), Sp1 (58), and E1A (111,123). This ability to physically interact with a wide array of proteins contributes to recognition of multiple target genes and imparts YY-1's characteristic multifunctionality. Thus, the promoter context, and/or pre-existing YY-1-protein complexes will dictate whether YY-1 behaves as a transcriptional activator, repressor, or initiator (20,36,68,96,123).

YY-1 activates the promoters of numerous genes including ribosomal proteins, the immunoglobulin heavy chain, dihydrofolate reductase, c-myc, and three viral

promoters. Although the mechanism(s) are still poorly elucidated, direct association between YY-1 and TFIIB, TBP, or TAFII55 have been demonstrated and this could play a role in the ability for YY-1 to initiate transcription and behave as a direct activator (6,113). Alternatively, YY-1 is known to interact with coactivators such as CREB binding protein (CBP) and adenovirus p300 which possesses HAT activity. This result suggests that recruitment of chromatin modifying enzymes could be yet another approach employed by YY-1 to transactivate promoters (120). Finally, structure/function analyses have identified a putative small transactivation domain within the carboxyl terminal domain that seems to behave as an activation/repression switch. This model proposes that when another factor interacts with the carboxyl terminal domain, it induces a conformational change in YY-1 that exposes the amino terminal transactivation domain effectively transforming it into a constitutive activator. Consistent with this model is the finding that YY-1 alone will repress an artificial promoter, but will activate it when in the presence of E1A which is known to bind to the switch region (120). It is noteworthy that these models are not necessarily mutually exclusive and they could be employed in combination depending on the target gene.

The repression function has been mapped to two domains within the first two zinc fingers of the carboxyl terminus and between residues 170 to 200 within the amino terminus (13,38,120). Several models have been proposed for YY-1-mediated repression. YY-1 could displace an activator by binding to overlapping DNA elements or induce bending that unfavorably alters the topology and spatial arrangement between activators and components of the transcription apparatus (55,113,120). In the quenching model, YY-1 disrupts communication between gene-specific activators and the general

transcription machinery, or interferes with the activator's target by binding to it itself (38,120). Lastly, YY-1 can recruit corepressors such as histone deacetylase which alter the local chromatin structure and thus restrict binding of cellular factors that positively regulate transcription (96,120).

The initiator function of YY-1 was initially characterized by studies on the AAV P5 promoter. Using a minimal *in vitro* reconstituted system, it was found that YY-1, TFIIB, and the RNA polymerase were sufficient to stimulate transcription. Since TATA binding protein (TBP) is generally considered essential for assembly of the preinitiation complex, it was postulated that YY-1 could substitute for TBP. In terms of the *in vivo* significance, these results raise the possibility that YY-1 could engage transcription in the absence of TFIID and a TATA box (123). However, there is evidence that the strength of YY-1-induced initiation *in vivo* is greatly improved by the presence of a TATA motif and interactions with TBP (113,120) or Sp1 which can recruit TFIID (36,58).

YY-1 influences HIV replication at several distinct stages. It recognizes the HIV-1 LTR initiator element and represses gene expression, and virus production (68). Later studies were able to duplicate these results and demonstrate that YY-1 forms a complex with LSF to synergistically inhibit the LTR by a mechanism that involves HDAC recruitment (20,96). YY-1 may inhibit efficient uncoating and maturation by interaction with cyclophilin A which binds to gag proteins and is specifically incorporated into HIV virions (74). Finally, overexpression of YY-1 can block HIV cell entry by downregulating expression of the co-receptor, CXCR4 (74).

2.4 c-MYC

c-Myc was first described in 1982 as the cellular homologue to the transforming sequences of the avian myelocytomatosis retrovirus, MC29 (23,64,88). The c-myc oncoprotein belongs to a larger family of related genes including USF, N-myc, L-myc, v-myc, B-myc, and S-myc (23).

The gene is located on human chromosome 8 and consists of only three exons, a long untranslated first exon and two exons representing the protein coding sequences. While there are two promoter regions termed P1 and P2, the majority of transcripts initiate from P2 to yield a protein with an apparent molecular weight of 64 kDa.

The amino terminus (NTD) is believed to be responsible for c-myc's multifaceted role in cell biology. Recently, a vast number of proteins have been shown to bind to this region including α-tubulin, Bin1, MM-1, pam, TRRAP, and Amy-1 (reviewed in reference 104). In addition, it includes two evolutionary conserved elements, Myc Box I and II (MBI, MBII) which are essential for all of c-myc's biological activities. The MBI domain (aa 45-63) was initially implicated in transactivation by using fragments of c-Myc protein fused to the DNA binding domain of the yeast Gal4 protein (44). In biological assays, it was found that MBI deletion mutants would diminish promoter activity and attenuate myc transformation. The MBI domain is the most prominently displayed region which coincides with its function as the principal site of phosphorylation that regulates transcription, and transformation. In particular, Threonine 58 (T58) is frequently mutated in Burkitts lymphoma which promotes protein stabilization by preventing efficient ubiquitination. This residue can also exist in three states: unmodified,

phosphorylated, and glycosylated. The impact of each of these states on myc protein stability and biological activity is yet to be fully addressed. MBII (aa 128-143) contains a PEST sequence necessary for rapid protein turnover, but not ubiquitination (reviewed in references 44,79,104). Furthermore, it was shown to be required for myc induced repression of the adenovirus major late promoter, and the growth arrest gene, gas1 (62,79). Whereas the region located between these elements defined by residues 92 to 106 were imperative for repression of cyclin D1 mRNA (89).

The carboxyl terminal domain (CTD) spans amino acids 360 to 439 and is comprised of a basic-Helix-Loop-Helix/Leucine Zipper (bHLH/LZ) motif. The basic region facilitates DNA binding, while the Helix-Loop-Helix participates in protein-protein interactions with Max, YY-1, AP-2, BRCA-1, TFII-I, Miz-1, and nmi (reviewed in references 23,44,64,65,79,104). A schematic representation of the c-Myc interacting proteins and their relative binding positions on c-Myc are shown in the upper diagram of Figure 7.

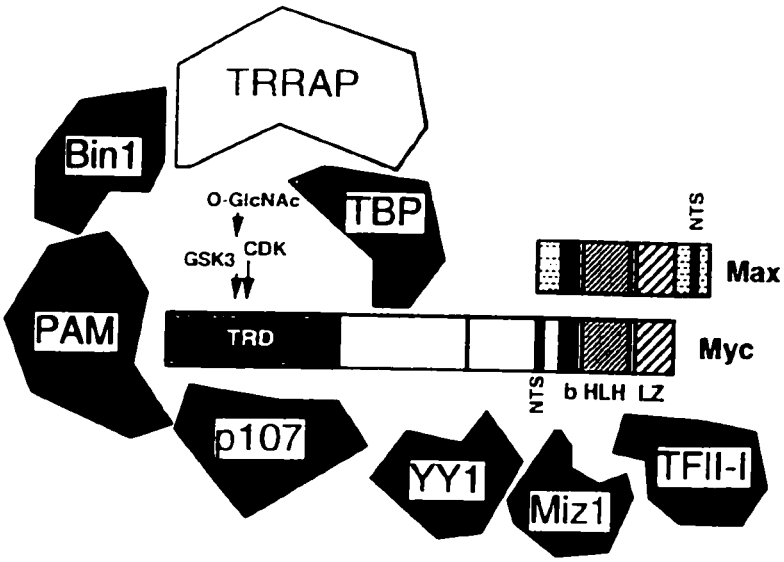
Through its function as a transcription factor, c-myc is able to regulate such disparate biological activities that includes growth (23,34,44,69,79), cell cycle progression (23,34,44,77,79,89), differentiation, tumorigenesis, genomic instability (44,79), cellular transformation (18,23,26,31,34,44,79), angiogenesis (44,79), and apoptosis (23,44,47,79,90). The current paradigm is that c-myc responds to signals from the cellular microenvironment, and regulates a specific subset of target genes. For this reason, c-myc is often referred to as "the intracellular sentinel of the extracellular milieu" (79).

c-Myc-mediated transactivation requires interaction with another bHLH class

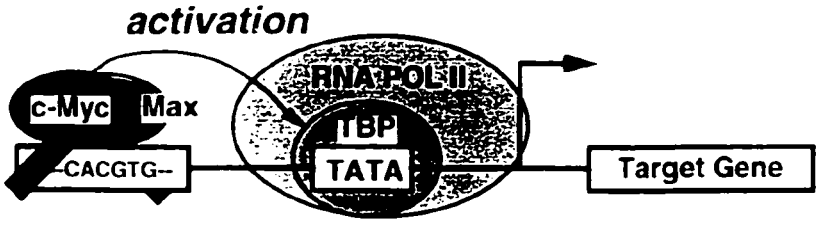
Figure 7: **c-Myc structure and its role in transcriptional regulation**

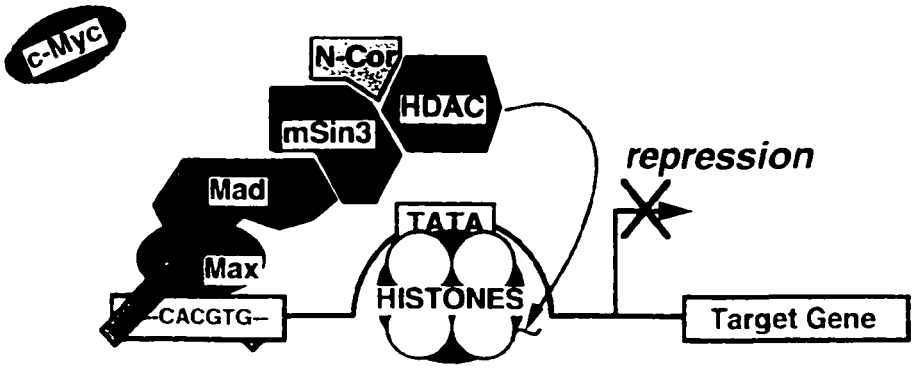
A schematic representation of cellular factors that associate with functional domains of the c-Myc protein are illustrated in A. Shown below are current models of c-Myc-Max transcriptional activation and repression. The c-Myc-Max heterodimer binds to canonical E box motifs and activates transcription via contacts made between c-Myc and TBP. The bottom diagram depicts the association of Mad-Max heterodimer with the E box followed by recruitment of co-repressors, mSin3, N-Cor, and histone deacetylase (23).

(a)


TRRAP
Bin1
O-GlcNAc
GSK3
CDK
TBP
NTS
Max
PAM
TRD
Myc
NTS
b HLH LZ
p107
YY1
Miz1
TFII-I

(b)


activation
c-Myc
Max
RNA POL II
TBP
TATA
CACGTG
Target Gene


c-Myc
N-Cor
HDAC
mSin3
Mad
Max
repression
TATA
HISTONES
CACGTG
Target Gene

protein known as Max (23,44,57,77,79,104). Unlike c-myc which is an unstable, and short-lived nuclear phosphoprotein, cellular levels of Max are greater and more constant in resting and proliferating cells. The transcriptional effects of c-myc and Max are distinct and depend upon the relative levels of each factor. In resting cells, Max is present in excess and can either form homodimers or interact with Mad family proteins which recruit corepressors such as mSin3 and histone deacetylase (57). Upon cellular stimulation, c-myc levels rise and outcompete Mad for heterodimerization with Max. c-Myc-Max complexes can then bind canonical E box motifs (5' CA(C/T)GTG 3') and transactivate the target gene via the c-myc amino terminus (44,57). The role of the Myc/Mad/Max network on transcription is represented as a schematic diagram in Figure 7. The mechanisms are not fully understood, but could involve nucleosome remodeling, association with members of the basal transcriptional machinery such as TBP (66,104), and/or recruitment of coactivators (3,44). In light of this finding, a symmetrical picture of the roles played by myc and Mad begins to emerge deduced from their temporal expression and differential recruitment of chromatin remodeling enzymes with opposing functions (3,44,79). It should also be mentioned that while there is evidence that c-myc can interact with TRRAP (104) which is linked to a histone acetyltransferase, promoter-bound myc has never been shown to influence the amount of acetylated histones. Recent chromatin immunoprecipitation studies of c-myc at the cad promoter identified high levels of histone acetylation in the G0 phase of the cell cycle and in differentiated cells when c-myc levels are low (11,29). Thus, recruitment of c-myc does not necessarily alter chromatin structure. These studies were integral in establishing a novel mechanism for c-myc-facilitated activation that involves stimulation of promoter clearance and elongation

via recruitment of P-TEFb (30). Alternatively, it was proposed that Myc-Max heterodimers are not bona fide activators at all because they only weakly transactivate (4- to10-fold) target promoters. Instead, Myc and Max could behave as derepressors by altering the local chromatin architecture to facilitate access of more potent constitutive transcription factors such as USF (44).

Repression is thought to be one of the few Max-independent functions of myc. Currently, this paradigm is under scrutiny due largely to chromatin immunoprecipitation studies that have identified Myc-Max heterodimers at repressed promoters (77,79). C-Myc-mediated repression was first demonstrated for the adenovirus major-late promoter via a mechanism that was contingent upon MBII, the bHLH domain, and the initiator element (62). Subsequently, numerous genes negatively regulated by c-myc were identified including c-myc itself (33,64,85,86), cyclin D1 (89), and collagen (127). A common thread underlying each of these mechanisms is c-myc's presence at the initiator. These DNA sequences are recognized by numerous cellular factors including YY-1, Miz-1, and TFII-I which also happen to associate with the bHLH domain belonging to c-myc (reviewed in 3,44,79,88,103,104). c-Myc utilizes multiple strategies to repress transcription that is dependent upon the nature of the interacting protein and the characteristics of the promoter. The most convincing data to support myc-mediated repression is the finding that c-myc could counteract transactivation by Miz-1 by forming ternary complexes that sequestered Miz-1 and increased c-myc stability (3,44,88). Recently, it was demonstrated that c-myc could repress the promoter for the cyclin-dependent inhibitor, p21 and thus promote G1 progression by sequestering the Sp1/Sp3 complex (42). c-Myc can also interact with TFII-I and obstruct TFII-I-TBP complex

formation (103). c-Myc can interact with YY-1 and inhibit it, whether YY-1 is behaving as an activator, or repressor (112).The mechanism precludes YY-1 interactions with components of the basal transcriptional machinery such as TBP and TFIIB, but does not block DNA binding (114). Interestingly, YY-1 can activate the c-myc P2 promoter (92) and inhibit its transforming ability in a manner that does not require physical interaction (7). Analyses of the repressed genes gadd45 (69), and PDGFRβ (78) have provided evidence for an initiator-independent mechanism whereby c-myc downregulates expression of CCAAT-dependent promoters by associating with the activator, CTF/NF-1 and suppressing its activity (64,78). Finally, on the basis of the accumulated evidence, there exists the distinct possibility that c-myc may not be an active transcriptional repressor since downregulation of gene expression in the presence of c-myc seems to occur by a mechanism that involves interference with transactivators rather than recruitment, and interaction with co-repressors (3).

3. Project Rationale

Regulation of HIV-1 proviral gene expression is intricately controlled by the binding of cellular transcription factors to cis-acting DNA elements within the long terminal repeat (20,40,48,96,100,129). Since the HIV-1 promoter possesses several putative c-myc binding sites including multiple E box motifs and initiator elements, we wanted to investigate the regulatory role of c-myc on HIV-1 LTR expression and determine if the c-myc effect is direct or indirect.

c-Myc is a member of the basic-helix-loop-helix/leucine zipper (bHLH/LZ) family of proteins (44,62). As such, it possesses all of the hallmarks of a transcription factor that enables it to regulate numerous cellular processes including proliferation, apoptosis, differentiation, and tumorigenesis (44,90). c-Myc activates transcription of target promoters by binding to E box motifs as a Myc-Max heterodimer (44,57,64). While the inhibitory mechanism of c-myc is more poorly characterized, current evidence supports an involvement of initiator elements (62) and binding proteins such as YY-1 (112-114) and TFII-I (103).

YY-1 is a ubiquitously expressed, multifunctional protein that can behave as an activator, repressor, or initiator depending on the promoter context (7,112-114). A simple model to explain the versatility of YY-1 is that interactions with disparate cellular proteins alter its activity. The human factors, YY-1 and LSF have been shown to form a multiprotein complex at the HIV-1 initiator element within the core promoter and synergistically downregulate gene expression (20,96). It has also been reported that YY-1 can physically associate with c-myc and that YY-1 activity is inhibited by this interaction

(112,113,114,131). Furthermore, c-myc transcription is negatively autoregulated by c-myc, and activated by YY-1 in a manner that is dependent upon their relative amounts (114). Thus, we hypothesize that one way c-myc may regulate transcription of the HIV-1 promoter is by modulating YY-1 activity.

In addition, there is evidence that c-myc interacts with TFII-I at initiator elements which leads to inhibition of transcription initiation by preventing the association of TFII-I and TATA binding protein (103). TFII-I also interacts with a member of the c-myc family, USF at the HIV-1 initiator and stimulates transcription (22). Therefore, the antagonistic activities of these factors may play an integral role in modulating HIV-1 gene expression.

Taken together, the HIV-1-LTR provides an intriguing model system to study the molecular, and functional interactions of both positive, and negative cellular factors and thus could have important implications in establishing novel therapeutic strategies.

4. Materials and Methods

4.1 Cell Culture

Two separate cell lines were employed. Jurkat cells are an immortalized human CD4+ T cell line, while 293-T cells are derived from human kidney epithelial cells.

Jurkat cells were grown in suspension in RPMI 1640 (Roswell Park Memorial Institute) containing 10% [v/v] fetal calf serum, penicillin [100U/mL], streptomycin [100ug/mL], glutamine [2mM], pyruvate [1mM], and HEPES [10mM]. Cells were maintained at 37^0C with 5% CO_2 . Transfections were performed when densities reached 1 million cells/mL of culture volume.

293-T cells were grown in monolayer in DMEM [Dulbecco's Modified Eagles Medium] supplemented with 10% [v/v] fetal calf serum, penicillin [100U/mL], and streptomycin [100μg/mL]. Cells were transfected when densities reached 50% of confluence.

4.2 Expression Vectors

The plasmid, HIV-1-LTR-CAT contains coding sequences for the U3 and R regions from the 5' long terminal repeat of the HXB2 clone of HIV-1. These regions are inserted upstream of the bacterial chloramphenicol acetyltransferase (CAT) gene of pSVO-CAT. The HIV-1-LTR linker scanning mutants are derivatives of the wild type promoter with the regions –21 to -4 and –3 to +15 sequences being replaced by an 18 base pair sequence corresponding to the restriction enzymes, NdeI-XhoI-SalI [CATATGCTCGAGGTCGAC]. These HIV-1-LTR plasmids were obtained from Dr.

Steven Zeichner at the NIH AIDS Research and Reference Reagent Repository. pSV-Tat is an SV40 driven tat gene required for transactivation of the HIV-1-LTR. pEF-c-Myc is a plasmid construct containing the coding exons for the wildtype c-Myc gene driven by the elongation factor promoter (a gift from Dr. Moulay Jamali, McGill University). The c-Myc amino terminal transactivation deletion mutants spanning amino acid 1 to 143 were cloned into the vector pMNBabeGFP-IRES and are driven by the rous sarcoma virus long terminal repeats (a gift from Dr. Linda Penn, University of Toronto). The plasmids, pCMV-YY-1, and pCMV-LSF represent the coding sequences for the genes driven by the cytomegalovirus promoter (a gift from Dr. Ulla Hansen, Boston University). pCMV-USF is a plasmid construct containing the USF gene cloned into the vector, pCDNA3.1(-) downstream of the cytomegalovirus promoter (a gift from Dr Robert Roeder, Rockefeller University). Virus production studies were performed with pBH10, the infectious molecular cDNA clone of HIV-1 that was obtained from the NIH AIDS Research and Reference Reagent Repository. pCMV-GFP is a plasmid containing green fluorescent protein driven by the cytomegalovirus promoter and was employed as an internal transfection control.

All plasmids were transformed in E.coli DH5α cells and selected for ampicillin resistance.

4.3 Transfections

Jurkat cells were transiently cotransfected by the DEAE-dextran method. Cells were subcultured one day prior and density was adjusted such that on the day of

transfection, cells were growing in active log phase. 15 million cells per transfection were suspended in 1mL of freshly prepared Tris-buffered saline (TS) containing 100 μg/mL each of $CaCl_2$ and $MgCl_2$ and 10 mM glucose. The DNA mixture was prepared by combining 1.0 μg of HIV-1-LTR-CAT and 0.25ug of pSV-Tat and 1.0 μg each of the transcription factor plasmids, pEF-c-Myc, pCMV-YY-1, pCMV-LSF, pCMV-USF in various combinations and 1mg/mL [v/v] of DEAE-dextran. In control transfections, 1.0 μg of the reporter construct is combined with 0.25 μg of pSV-Tat and the unmodified vector(s) for each gene of interest. All transfections were adjusted with an appropriate quantity of salmon sperm DNA. The cell suspension was then combined with the DNA-DEAE-dextran mixture and incubated for 60 minutes at 37^0C with 5% $C0_2$. 10% Dimethylsulfoxide (DMSO) [v/v] was added for 3 minutes to enhance transfection efficiency followed by immediate dilution in phosphate-buffered-saline (PBS) [Ca^{2+}/Mg^{2+} free]. Cells were washed twice with PBS [Ca^{2+}/Mg^{2+} free] before being resuspended in complete medium and incubated for 48 hours at 37^0C with 5% $C0_2$ (reviewed in 106).

293-T cells were transiently cotransfected by the $CaCl_2$-HEPES-Phosphate precipitation method. Briefly, 5 x 10^5 cells were plated in 35mm^2 tissue culture dishes one day prior to transfection. The DNA mixture was prepared as previously described to a total volume of 500uL in sterile ultrapure water and adjusted to 10.0 μg with salmon sperm DNA. The DNA mixture was incubated with 2.5M $CaCl_2$ for 15 minutes at room temperature (RT). The DNA was then precipitated by the subsequent addition of 2X HEPES-buffered-saline (HBS) solution and added dropwise to the cell monolayer. 16 hours post-transfection, fresh culture medium was added and the cells continued to be

incubated for a total of 48 hours at 37^{0}C with 5% $C0_2$ (reviewed in 107).

4.4 Chloramphenicol Acetyl Transferase Assay

Cells were harvested 48 hours posttransfection. Cells were washed once in 5 mL of PBS followed by a more stringent wash in TEN buffer (40 mM Tris [pH 7.5], 1 mM EDTA [pH 8.0], 15 ηM NaCl). Cell pellets were recovered by high speed centrifugation and resuspended in 0.25 M Tris [pH 7.5]. Cells were then lysed by 3 consecutive cycles of freezing and thawing. The supernatants were collected and analyzed for protein content by the modified Lowry method. Approximately 100-150 μg of protein was incubated for 60 minutes at 37^{0}C with 4 mM acetyl CoA, and 0.1 μCi of [1,2-^{14}C]chloramphenicol. The chloramphenicol metabolites were extracted with the organic solvent, ethyl acetate and then spotted on thin-layer chromatography (TLC) silica gel membranes. The various metabolites were resolved by a 19:1 eluant of chloroform:methanol and analyzed by exposure to a Bio-Rad GS 250 phosphor-Image screen for 24 hours. The results were visualized using the Bio-Rad GS-363 scanner and the chloramphenicol metabolites were then quantitated using Bio-Rad Molecular Analyst software (version 2.0.1). The level of CAT expression was calculated as the percentage of the two acetylated forms of chloramphenicol relative to the total amount of [1,2-^{14}C]chloramphenicol (43,110, reviewed in 105). Reporter assays were repeated with more or less cell lysate if the percent CAT activity was not within the acceptable linear range of 5 to 70%. Transfection efficiencies in Jurkat cells were normalized by flow cytometry using pCMV-GFP as an internal control, while transfection levels in 293-T cells were

measured with a cmv-β-galactosidase plasmid. Experiments were repeated at least three times to estimate the variations of transfection efficiency.

4.5 HIV-1 Reverse Transcriptase Assay

Cells were transfected with 1.0 μg of pBH10 and 1.0 μg each with the transcription factors pEF-c-myc, pCMV-YY-1, pCMV-LSF in various combinations as previously described. 24, 48, and 72 hours post-transfection, cell suspensions were cleared by low speed centrifugation and culture supernatants were analyzed for the level of virus production. A RT master mix (50 mM Tris-Cl pH 7.8, 75 mM KCl, 2 mM DTT, 5mM $MgCl_2$, 5 μg/mL polyA, 6.25 μg/mL oligodT, 0.5% (v/v) NP-40) was radiolabelled with 10 mCi/mL [^{3}H-dTTP] and aliquoted to a 96 well microtiter plate. To this, 10 μL of recovered supernatant was added and incubated for 2 hours at 37^{0}C. The labeled supernatant was spotted on to Whatman DE81 paper and dried for 10 minutes. The DE81 paper was washed five times, and dried. The radioactive spots were cut out and counted in a scintillation counter. The total number of counts per minute (cpm) is directly proportional to the amount of reverse transcriptase enzyme and therefore virus present (59).

4.6 Preparation of Nuclear Extracts

10 million cells were collected by low speed centrifugation for 5 minutes. The cell pellet was washed with PBS, centrifuged and resuspended in pre-chilled buffer A

containing 10 mM HEPES [pH 7.0], 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, and 0.5 mM PMSF. The supernatant was cleared by centrifugation and the cell pellet was resuspended in buffer A/0.1% NP-40 and incubated on ice for 10 minutes to permit cell lysis. The cytoplasmic fraction (i.e. the supernatant) was collected by a 10 minute centrifugation at 14,000 rpm. The pellet was further resuspended in chilled buffer B (containing 20 mM HEPES [pH 7.9], 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 5 μg/mL leupeptin, 5 μg/mL pepstatin, 5 μg/mL aprotinin, 0.5 mM spermidine) and incubated for 15 minutes on ice. The nuclear proteins were subsequently collected by high speed centrifugation and diluted in 75 μL of cold buffer C (20 mM HEPES [pH 7.0], 20% glycerol, 0.2 mM EDTA, 50 mM KCl, 0.5 mM DTT, 0.5 mM PMSF) (15,41). Protein content was determined by the Bradford assay.

4.7 Electrophoretic Mobility Shift Assay

The double stranded DNA probe used in the binding reactions represents the -19 to +26 region of the HIV-1-LTR initiator element (5'TGCTTTTTGCCTGTACTGGGTCTCTCTGGTTAGACCAGATCTGAG-3') and contains binding sites for the transcription factors, USF, LSF, and YY-1. The oligonucleotide was end-labeled with [γ-^{32}P] and T4 polynucleotide kinase for 1 hour at 37C. The labeled oligonucleotides were purified on a G-25 sephadex column. Protein-DNA binding reactions consisted of 5 μg of nuclear extract, 0.2 ηg of radioactively end-labeled double stranded probe, 2.5 μg of poly(dI-dC) to reduce nonspecific interactions

and DNA binding assay (DBA) buffer (10 mM Tris [pH 7.5], 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 4% glycerol). In competition reactions, nuclear extracts were preincubated with a 100-fold excess of unlabelled oligonucleotide. In antibody depletion experiments, DNA binding reactions were supplemented with anti-c-myc antibody, a nonspecific antibody, or pre-immune serum. The reactions were incubated for 30 minutes at room temperature. The DNA-protein complexes were resolved on a 5% nondenaturing polyacrylamide gel (60:1). Electrophoresis was performed using the Protean II xi from Bio-Rad in 0.5X Tris-borate-EDTA (TBE) at 200V for 4 hours in the cold. The gel was blotted on to 3MM Whatman filter paper and dried for 30 minutes at 80^0C before being exposed to Kodak X-Omat Blue film (15,41).

4.8 Whole Cell Extracts

10 million cells were collected by low speed centrifugation and lysed in 1 mL of RIPA buffer (50 mM Tris [pH 7.5], 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM AEBSF, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin, 1 mM Na_3Va_4 and 1 mM NaF) for 15 minutes at 4^0C. The lysate was cleared by centrifugation at 14,000 rpm for 30 minutes. For co-immunoprecipitation studies, the lysate was incubated with 100 μL of protein A sepharose (50% slurry) at 4^0C for 10 minutes on an orbital shaker (reviewed in 108). Proteins were quantified by the modified Lowry method and adjusted to a concentration of 1μg/uL with PBS.

4.9 Co-Immunoprecipitation

500 µg of whole cell extract was tumbled overnight at 4°C with the rabbit polyclonal antibodies for c-Myc or YY-1 or LSF, or a non-specific rabbit IgG as a control. The antibody-protein complexes were precipitated by the addition of 100 µL of a 1:1 protein A sepharose slurry in PBS and allowed to tumble at 4°C for 3 hours. The antibody-protein complexes were washed 3 times in PBS to remove any non-specific proteins. 40 µL of 2X Laemmli buffer (containing 0.2 M Tris-Cl [pH 6.8], 4% SDS, 20% glycerol, 0.008% bromophenol blue and 8% β-Mercaptoethanol) was added to the beads and incubated at 95°C for 5 minutes. Supernatants were recovered by pulse centrifugation (reviewed in 108).

4.10 Western Immunoblotting

Proteins were applied to a 10% SDS-polyacrylamide gel (37.5:1) and electrophoresed in 1X Tris-Glycine buffer at 200 V for 40 minutes. The proteins were subsequently electrophoretically transferred for 1 hour at 100 V to a 0.2 µM pore nitrocellulose membrane. The membrane was blocked with 0.1% PBS-Tween-20 and 5% non-fat milk for 1 hour at room temperature or overnight at 4°C with rotation. The membrane is then incubated with a 1:1000 dilution of the primary antibody in the blocking reagent for one hour at room temperature or overnight at 4°C. The membrane was washed in 0.1% PBS-T and then incubated with a 1:1000 dilution of the secondary antibody conjugated to horse-radish peroxidase in blocking reagent for 30 minutes at

room temperature. The membrane is again washed prior to the addition of the chemiluminescence reagents (ECL, Amersham) and exposure to Kodak X-Omat Blue film for 1 minute (reviewed in 109).

4.11 Cell Viability

Cells were transfected as previously described. Cells were harvested 48 hours later, washed in phosphate-buffered-saline and resuspended at a density of 1 million cells per milliliter. Viability was determined by flow cytometric analysis in which the cell sub-populations were sorted according to their forward and side scatter patterns.

4.12 Transfection Efficiency

Transfection levels were determined by flow cytometry for Jurkat cells and the standard β-galactosidase assay for 293-T cells.

Briefly, 48 hours posttransfection, Jurkat cells were harvested by low-speed centrifugation and washed twice in Phosphate-buffered-saline. Cells were resuspended at a density of one million cells/mL and analyzed by the flow cytometer. The machine was calibrated using Jurkat cells that were not transfected with green-fluorescent protein in order to distinguish autofluorescent cells and bona fide transfected cells.

Cell extracts were prepared for 293T cells as previously described for the reporter assay. In a 96-well microtiter plate, a mixture containing 100X magnesium, 1X ONPG, 0.1 M sodium phosphate (pH 7.5) and 5 μL of cell extract was prepared. The reactions

were incubated at 37^0C until a faint yellow color developed and the optical density was determined at a wavelength of 450 ηm at two separate time points. The specific activity was subsequently calculated by subtracting the absorbance at time point one from the absorbance at time point two and dividing the result by the quantity of protein used per volume assayed (4).

4.13 Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 2 software. Results were analyzed by one-way analysis of variance (ANOVA) in which the lowest level of significance was set at $*p<0.05$ with a 95% confidence level.

RESULTS

5.1 The effect of c-myc titrations on tat-activated HIV-1 LTR-CAT expression

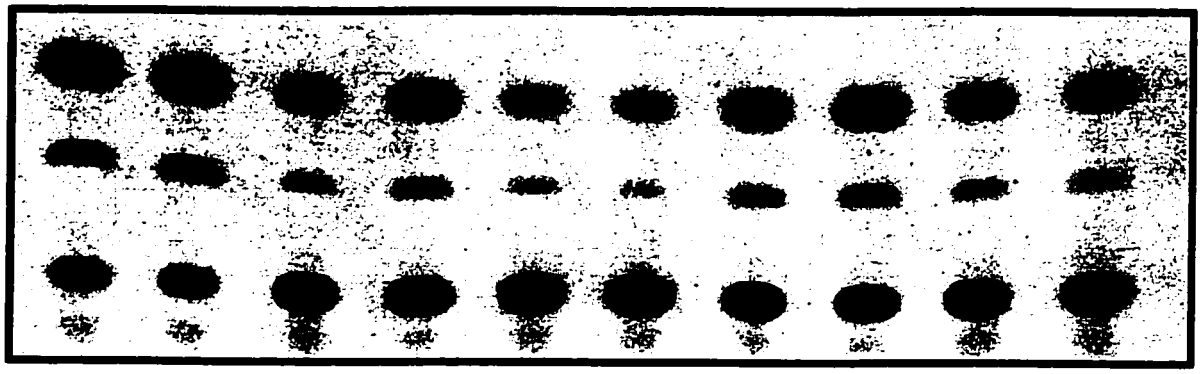
C-myc has been shown to activate or repress transcription. The ability for increasing amounts of ectopically expressed c-myc to decrease tat-activated HIV-1 LTR-directed expression of CAT was tested. Jurkat cells were transiently co-tranfected with the HIV-1 LTR-CAT reporter, tat, and 1.0 μg or 2.0 μg of c-myc driven by the human elongation factor promoter. In control experiments, cells were co-tranfected with the reporter, tat, and 1.0 μg or 2.0 μg of the unmodified vector. All transfections were supplemented with salmon sperm DNA to ensure that differences in HIV-1 LTR-CAT expression amongst the samples could not be attributed to variability in total DNA content. CAT assays were performed 48 hours post-transfection, were normalized for protein content and quantitated by phosphorimaging. The data in Figure 8 displays typical results of an autoradiograph of a thin layer chromatography membrane, while Figure 9 illustrates the accumulated data described as the average percent CAT expression in graphical form.

Exogenous expression of c-myc could inhibit tat-activated HIV-1 promoter activity in a dose-dependent manner. The population of cells that received 1.0 μg of c-myc repressed HIV-1 gene expression by 30.76% ± 2.96 relative to the control, while 2.0 μg of c-myc plasmid inhibited LTR-CAT activity by 43% ± 9.5 over its control. The results obtained represent the average of 5 independent experiments and the differences between treated and untreated samples was found to be statistically significant ($*p<0.05$) as determined by the one-way analysis of variance test.

Figure 8: **The effect of c-myc titrations on tat-activated HIV-1 LTR-CAT expression in Jurkat cells.**

Results of a representative autoradiograph of a thin layer chromatography membrane are shown. Populations of Jurkat cells were transiently cotransfected with the HIV-1 LTR-CAT reporter construct, tat, and 1.0 μg or 2.0 μg of c-myc or 1.0 μg or 2.0 μg of the unmodified vector as controls. The results are presented as duplicates. The bottom set of spots corresponds to unmetabolized chloramphenicol. The spots directly above represent chloramphenicol acetylated at the 1' hydroxyl group, while the uppermost spots depict chloramphenicol acetylated at the 3' position. The results shown have been normalized for protein content.

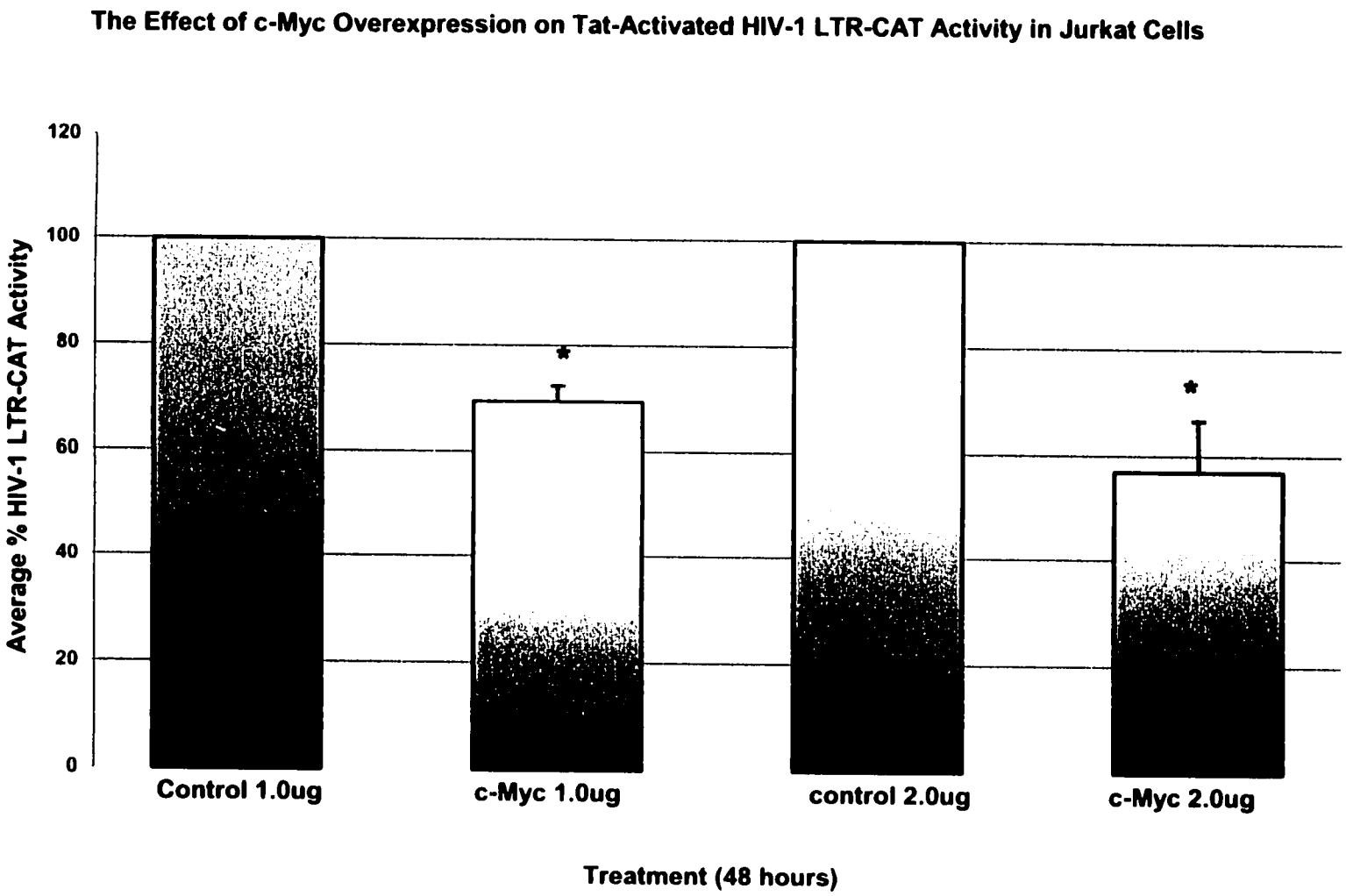
The Effect of c-Myc on Tat-activated HIV-1 LTR-CAT Expression In CD4+ T Cells



HIV-1 LTR-CAT +TAT	+c-Myc (1.0 μg)	+c-Myc (2.0 μg)	+Control (1.0 μg)	+Control (2.0 μg)

Figure 9: **Inhibition of LTR-directed CAT expression by c-myc in transfected Jurkat cells**

The expression of radioactive chloramphenicol was quantitated by phosphorimaging and the percent CAT activity was calculated as the amount of acetylated chloramphenicol divided by the total chloramphenicol. The average results of 5 independently performed experiments are expressed as +SEM in graphical form. Statistical significance was determined by one-way ANOVA, *$P<0.05$.


The Effect of c-Myc Overexpression on Tat-Activated HIV-1 LTR-CAT Activity in Jurkat Cells
Average % HIV-1 LTR-CAT Activity
120
100
80
60
40
20
0
*
*
Control 1.0ug
c-Myc 1.0ug
control 2.0ug
c-Myc 2.0ug
Treatment (48 hours)

5.2 The Effect of c-Myc and USF on Tat-Activated HIV-1 LTR-CAT Expression

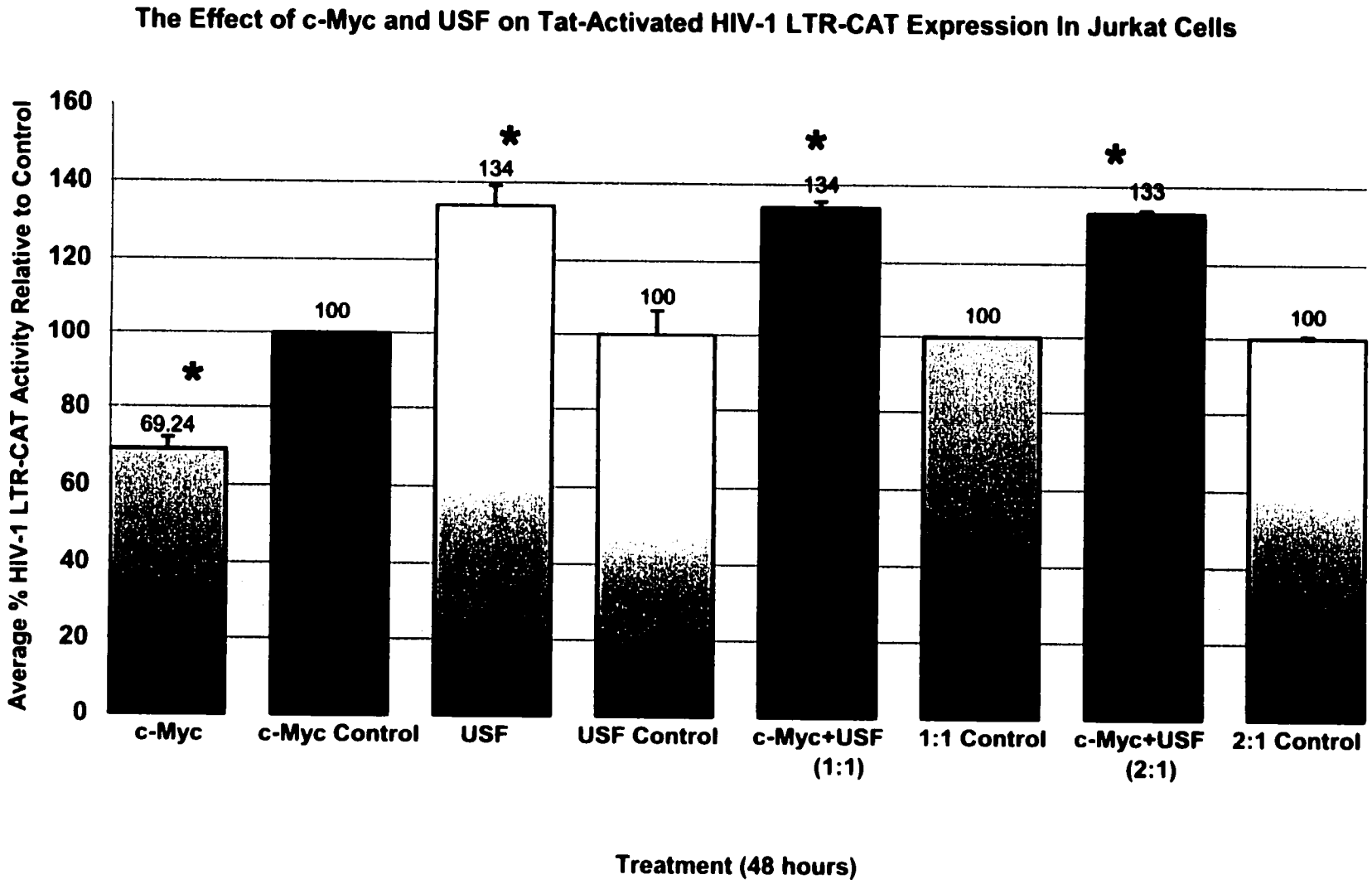
It was previously reported that c-Myc represses (62), while USF stimulates transcription through the initiator elements of the adenovirus major-late promoter (28). We therefore wanted to investigate the antagonistic relationship of these factors using the HIV-1 promoter as the model system.

Jurkat cells were transiently transfected with the HIV-1 LTR-CAT reporter construct, tat, and c-myc and/or USF as previously discussed. In control experiments, the cells were transfected with the appropriate unmodified vector. Reporter assays were conducted 48 hours post-transfection and results were quantitated by phosphorimaging analysis.

Figure 10 demonstrates the compiled results obtained in Jurkat cells. Cells transfected with 1.0 μg of c-Myc exhibited 31% ± 3 reduced LTR-CAT expression relative to the control, while cells that received 1.0 μg of USF plasmid enhanced gene expression by 34% ± 5.2 with respect to the unmodified vector control. To determine if the effects of c-Myc and USF on the HIV-1 LTR were competitive, additive, or synergistic, cells were transfected with increasing amounts of c-Myc in the presence of constant levels of USF. When c-myc and USF were co-expressed in a 1:1 ratio, LTR-CAT activity was increased by 34% ± 1.7 with respect to the control. Similarly, when c-Myc and USF were transfected in a 2:1 ratio, HIV-1 expression was heightened by 33% ± 0.75 with respect to the control. The results were determined to be statistically significant by the one-way ANOVA test, *$P<0.05$. Thus, USF-mediated stimulation of the HIV-1 LTR cannot be overcome by increasing amounts of c-Myc which implies that the

Figure 10: The effect of c-Myc and USF on tat-activated HIV-1 LTR-directed CAT expression in Jurkat cells

Jurkat cells were transiently transfected with the HIV-1 LTR-CAT reporter, tat, and combinations of c-Myc and USF as previously described. The results are presented graphically as the average percent CAT activity relative to control and are expressed as +SEM for n=3. Statistical significance was determined by one-way ANOVA, *$P<0.05$.


The Effect of c-Myc and USF on Tat-Activated HIV-1 LTR-CAT Expression In Jurkat Cells
Average % HIV-1 LTR-CAT Activity Relative to Control
160
140
120
100
80
60
40
20
0
*
69.24
100
*
134
100
*
134
100
*
133
100
c-Myc
c-Myc Control
USF
USF Control
c-Myc+USF (1:1)
1:1 Control
c-Myc+USF (2:1)
2:1 Control
Treatment (48 hours)

mechanism for c-Myc-facilitated repression does not occur by outcompeting, or interfering with this activator in Jurkat cells.

Figure 11 describes the average percent HIV-1 LTR-CAT activity results obtained in 293-T cells. Overexpression of c-Myc inhibited HIV-1 LTR-CAT activity by 24.1% ± 2 with respect to the control and was statistically significant (*$P<0.05$). Similarly, overexpression of USF resulted in reduced HIV-1 LTR expression by 21.6% ± 3.67, but it was not statistically different from the control. When both c-Myc and USF were co-expressed in a 1:1 and 2:1 ratio, HIV-1 LTR expression was significantly enhanced (**$P<0.001$) by 164% ± 3.13 and 147.1% ± 11 beyond the control, respectively. This indicates that the combined effects of these factors on HIV-1 LTR expression is synergistic. The discrepancy amongst the two cell types may be attributed to differences in transfection techniques, the presence of a positive factor, the lack of a negative factor, or the relative proportion of c-Myc and USF in the cell.

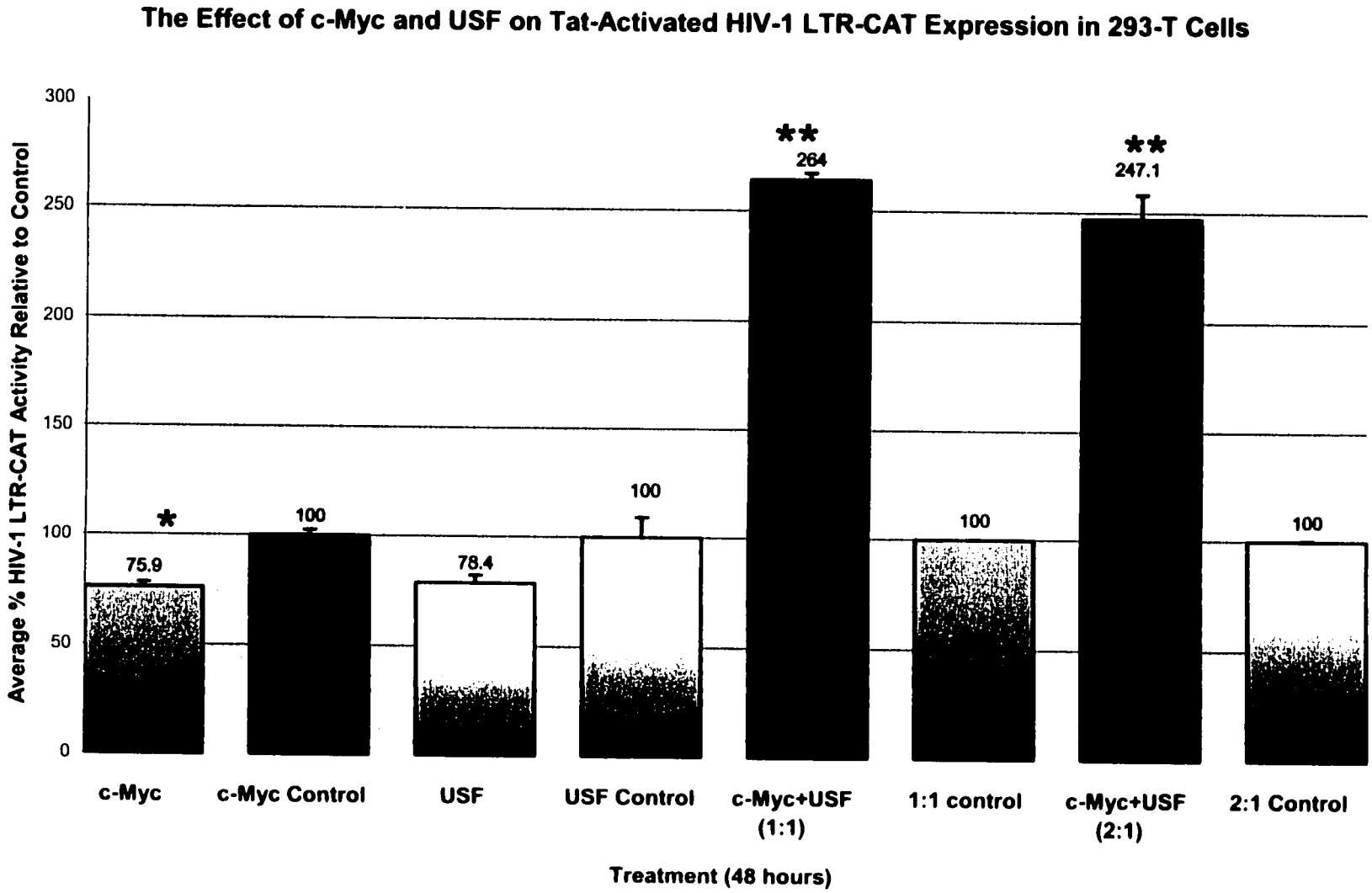
5.3 The effect of c-Myc, YY-1, and LSF on tat-activated HIV-1 LTR-CAT expression

Since it was shown that association with c-myc inhibits the transcriptional activating and repressing abilities of YY1 (114) and that YY1 and LSF could synergistically repress the HIV-1 LTR (20,96), we wanted to investigate the effect of overexpressed c-Myc on gene expression in the presence of YY1, and/or LSF.

A population of Jurkat cells was transiently co-transfected with the HIV-1 LTR-CAT reporter plasmid, tat, and various combinations of the transcription factors, c-Myc, YY-1, and LSF. In control experiments, the cells received the appropriate unmodified

Figure 11: The effect of c-Myc and USF on tat-activated HIV-1 LTR-CAT expression in 293-T cells

Cells were transfected with HIV-1 LTR-CAT, tat, and combinations of the factors c-Myc and USF as previously described. The results were determined by CAT analysis and are illustrated graphically as the average % CAT activity with respect to the control empty vector. The results are expressed as + SEM for n=3. Statistical significance was determined by the one-way ANOVA test, *P<0.05, **P<0.001.


The Effect of c-Myc and USF on Tat-Activated HIV-1 LTR-CAT Expression in 293-T Cells
Average % HIV-1 LTR-CAT Activity Relative to Control
300
250
200
150
100
50
0
*
**
**
75.9
100
78.4
100
264
247.1
100
100
c-Myc
c-Myc Control
USF
USF Control
c-Myc+USF (1:1)
1:1 control
c-Myc+USF (2:1)
2:1 Control
Treatment (48 hours)

vector for each transcription factor. A representative sample of an autoradiograph of a CAT assay is illustrated in Figure 12. The quantitation of the data can be seen in Figure 13. Cells that received 1.0 μg of pCMV-YY1 inhibited LTR expression by 31.84% ± 3.257 relative to its control. While co-overexpression of pCMV-YY1 and pCMV-LSF or pCMV-YY1 and pEF-c-Myc reduced HIV-1 LTR-CAT activity by 70.97% ± 7.945 and 52.82% ± 3.085, respectively. Overexpression of pCMV-LSF alone resulted in 34.5% ± 5.469 repression relative to its control. Cells that received 1.0ug each of pCMV-LSF and pEF-c-Myc inhibited the HIV-1 LTR by 74.87% ± 2.734 with respect to their control. When all three transcription factors were co-overexpressed with the HIV-1 LTR reporter in the presence of tat, expression was reduced by 79.3% ± 6.153. In all experiments, the differences between the treated and control groups was statistically significant (*$P<0.01$, **$P<0.001$) as determined by the one-way ANOVA. The effects of co-overexpression of c-myc, YY1, and LSF together on the HIV-1 LTR was not statistically significant when compared to any paired combination of transcription factors. However, reduced gene expression in the presence of all three factors was consistently observed.

5.4 The Effect of Transfection on Cell Viability

Since the effects of the transcription factors on HIV-1 LTR gene expression were relatively modest, it was necessary to confirm that repression was not simply due to transfection toxicity. Cell viability is used to determine the proportion of living and dead cells, although it does not distinguish amongst necrotic, or apoptotic-induced death. 48 hours post-transfection, cells were recovered and analyzed by flow cytometry. The data

Figure 12: The effect of c-Myc, YY1, and LSF on tat-activated HIV-1 LTR-directed CAT expression in Jurkat cells

A representative example of a thin layer chromatography membrane is shown. All populations of cells were transfected with the HIV-1 LTR-CAT reporter and tat or with 1.0 μg of c-myc, YY1, LSF alone or in various combinations. In control experiments, cells received the unmodified vector(s). While results were quantitated on the basis of their respective controls, for simplicity, only one control is presented here (the HIV-1 LTR-CAT + Tat).

The Effect of c-Myc, YY-1, and LSF on Tat-Activated HIV-1 LTR-CAT Directed Expression in CD4+ T Cells

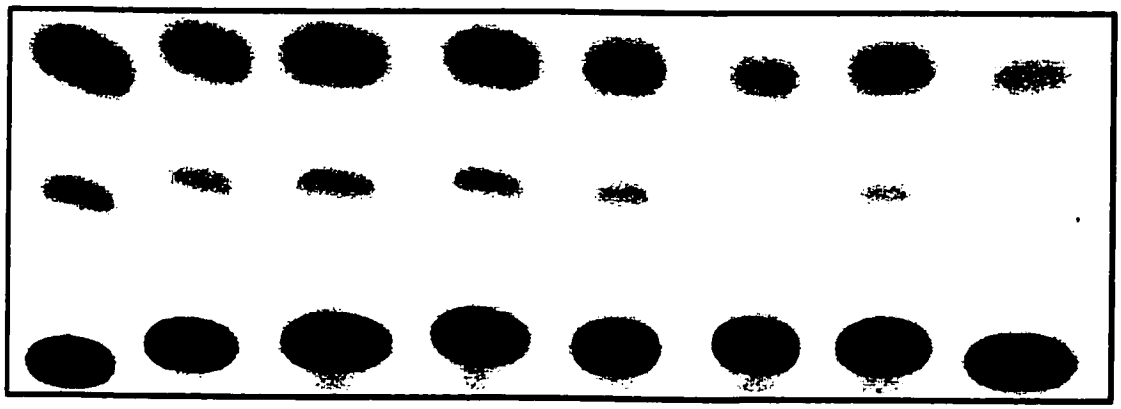
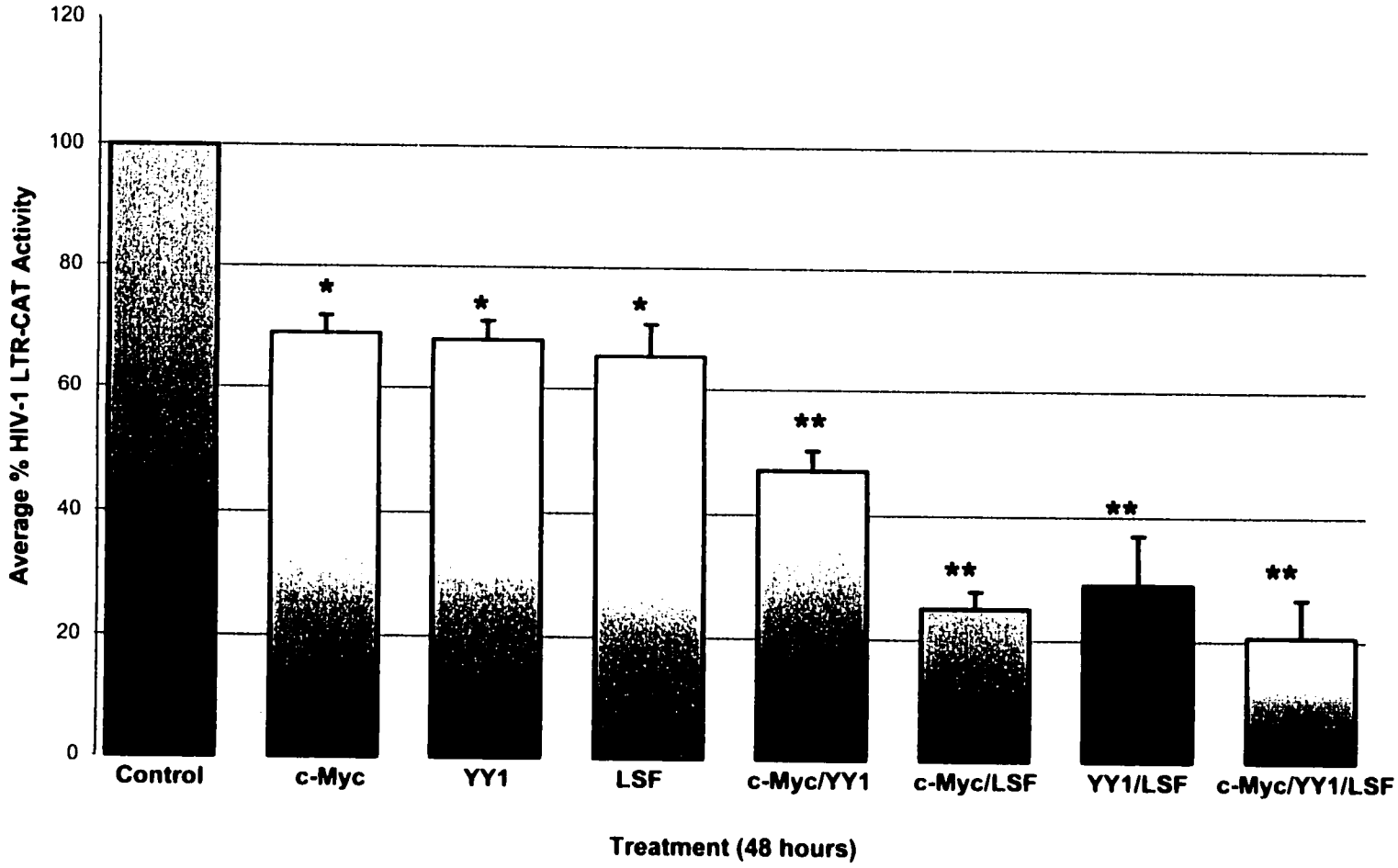


Figure 13: The effects of co-expression of c-myc, YY1 and LSF in transfected Jurkat cells

Jurkat cells were transfected and analyzed for CAT activity as previously discussed. The graph shows the average percent CAT activity of each group relative the control empty vector. The results are expressed as +SEM for n=5. Statistical significance was determined by the one-way ANOVA test (*P<0.01, **P<0.001).

The Effect of co-overexpression of c-myc,YY1 and LSF on Tat-Activated HIV-1 LTR-CAT Expression



in Figures 14 and 15 illustrate the results of three independently performed experiments in Jurkat and 293-T cells, respectively.

Overall, significant differences were not observed amongst cells that were transfected with c-Myc, YY-1, LSF, and USF compared to untransfected cells and their respective controls in both Jurkat and 293-T cell lines. This indicates that the effects of these factors on the HIV-1 LTR reporter is not due to toxicity as a result of the transfection techniques.

5.5 Western Immunoblotting Results

We sought evidence to confirm that the effect of the transcription factors, c-myc, YY-1, and LSF on HIV-1 LTR-CAT was due to their ectopic expression and also to determine if overexpression of one factor influenced the expression of other endogenous proteins. Jurkat and 293-T cells were transfected with c-Myc, YY-1, LSF, and tat as previously described. In mock experiments, cells were subjected to the transfection ritual without the addition of DNA. Cells were lysed to obtain nuclear extracts and proteins were separated by 10% SDS-PAGE. Immunoblots were probed for c-Myc, YY-1, LSF and B-actin expression. The results are presented in Figure 16 for Jurkat nuclear extracts and Figure 17 for 293-T nuclear extracts.

In Jurkat cells, c-Myc overexpression was not detectable in transfected cells compared to untransfected cells (Fig.16A, compare lane1 and 5). Furthermore, transfection of YY-1, LSF, and tat did not appear to influence the expression of endogenous levels of c-Myc (Fig.16A, compare lanes 2-4 and 5). Similarly, transfection of YY-1, and LSF did not result in enhanced expression relative to the controls (Fig.16B,

Figure 14: The effect of transfections of various factors on cell viability in Jurkat cells

Cells were transfected as previously described. Cell viability was determined 48 hours post-transfection by flow cytometry. The results are presented as the average percent cell viability expressed as + SEM for n=3.

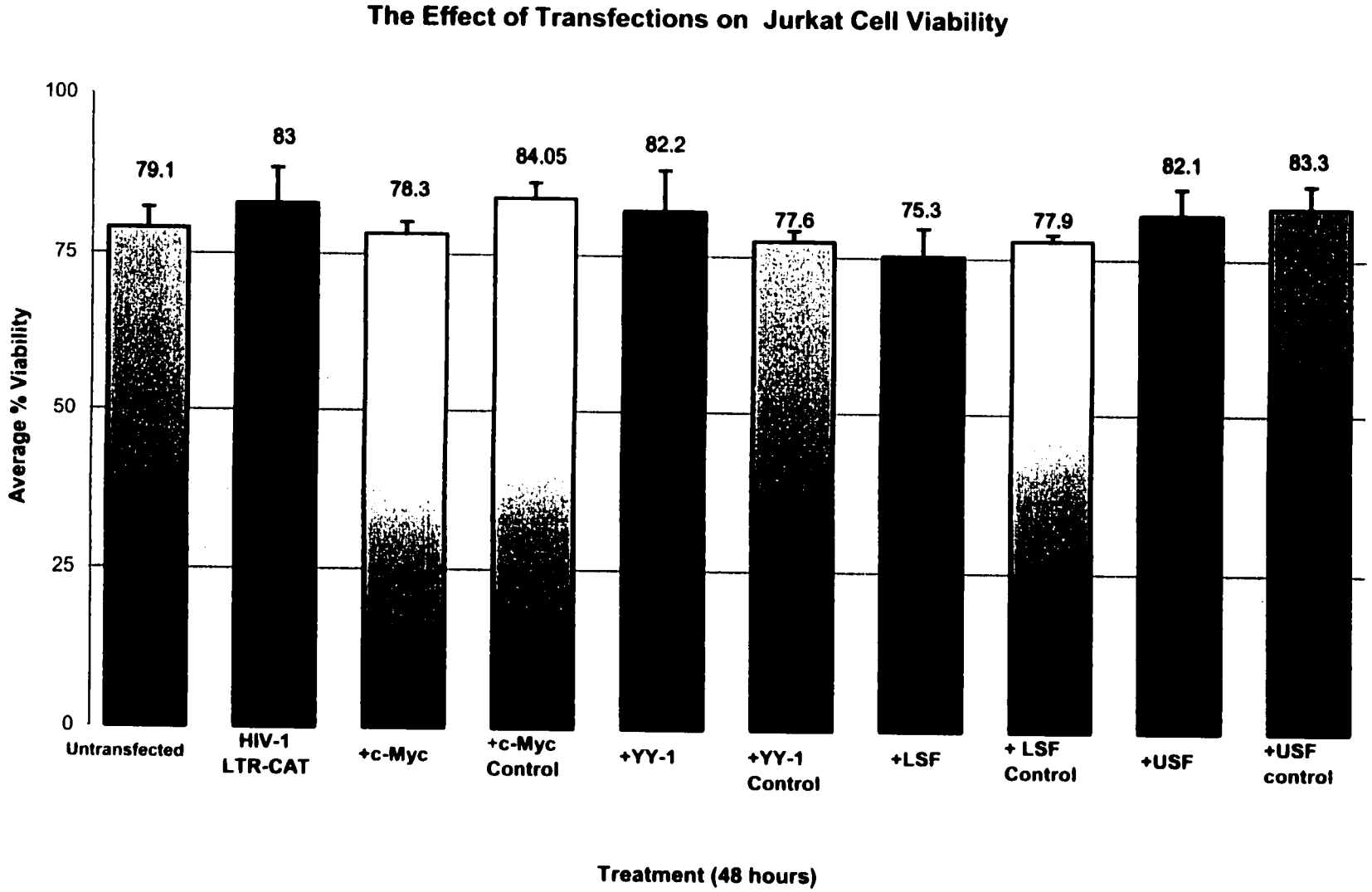

The Effect of Transfections on Jurkat Cell Viability
Average % Viability
100
75
50
25
0
79.1
83
78.3
84.05
82.2
77.6
75.3
77.9
82.1
83.3
Untransfected
HIV-1 LTR-CAT
+c-Myc
+c-Myc Control
+YY-1
+YY-1 Control
+LSF
+ LSF Control
+USF
+USF control
Treatment (48 hours)

Figure 15: The effect of transfection on 293-T cell viability

Cell viability was determined 48 hours post-transfection by flow cytometry. The results are shown graphically as the average percent cell viability expressed as +SEM for n=3.

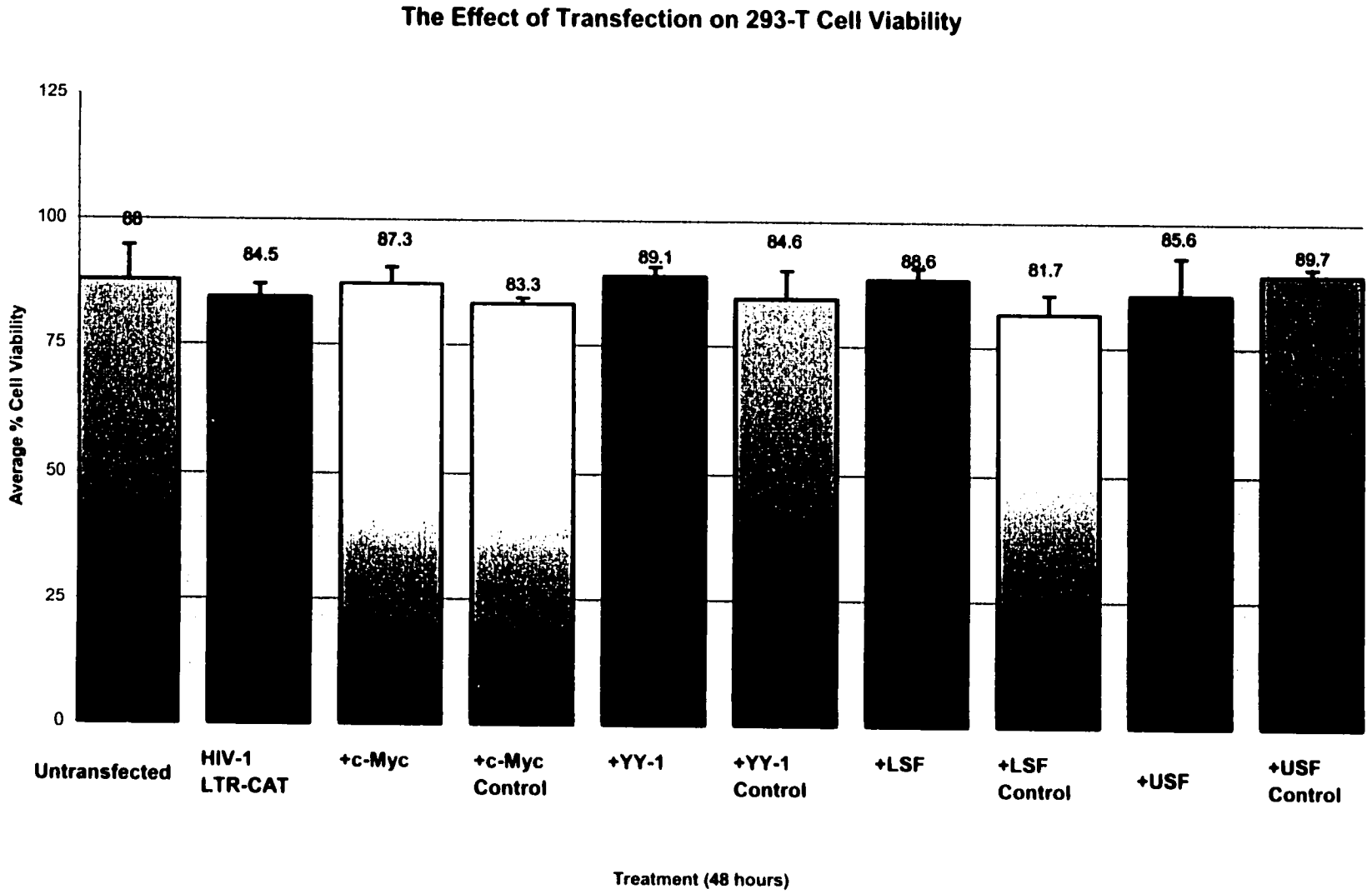

The Effect of Transfection on 293-T Cell Viability
Average % Cell Viability
125
100
75
50
25
0
88
84.5
87.3
83.3
89.1
84.6
88.6
81.7
85.6
89.7
Untransfected
HIV-1 LTR-CAT
+c-Myc
+c-Myc Control
+YY-1
+YY-1 Control
+LSF
+LSF Control
+USF
+USF Control
Treatment (48 hours)

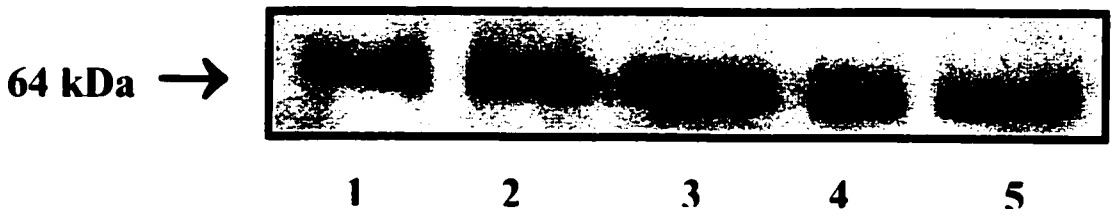
Figure 16: Western immunoblot analysis in transfected Jurkat cells

Cells were transfected as previously described. 10 μg of nuclear extract was electrophoresed, transferred to a support membrane and probed for A) c-Myc , B) YY-1 C) LSF and D) B-Actin expression. The lanes are designated as follows:

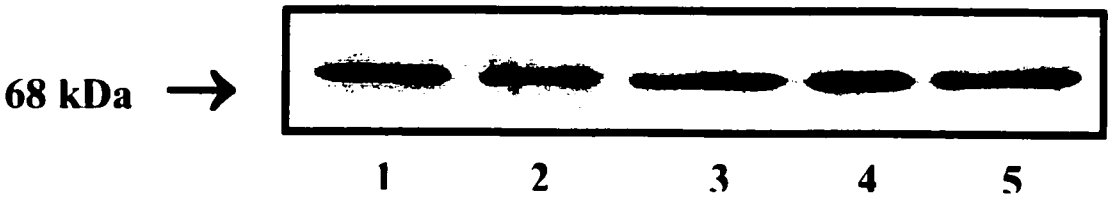
1) c-Myc (1.0 μg)
2) YY-1 (1.0 μg)
3) LSF (1.0 μg)
4) Tat (0.25 μg)
5) Mock

Western Blot Results In Transfected Jurkat Cells

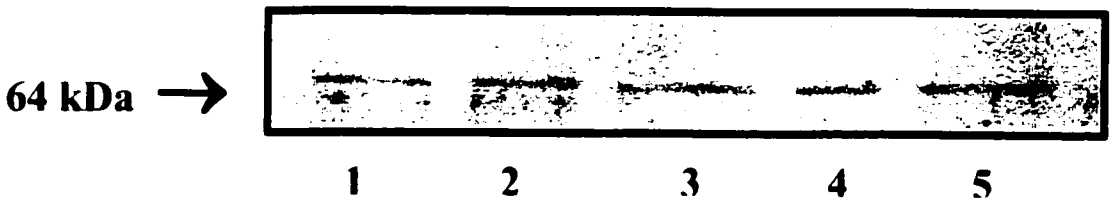
A) c-Myc



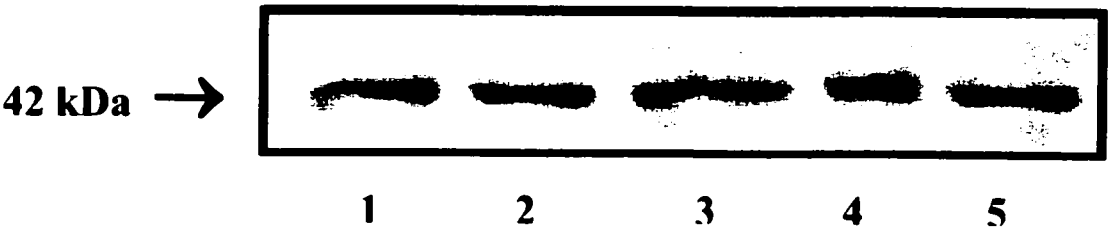
B) YY-1



C) LSF



D) B-Actin



compare lane 2 and 5; 16C, compare lane 3 and 5). The B-actin western was performed to confirm equal protein loading.

293-T cells were transfected to investigate cell type differences. Substantial overexpression of c-Myc, YY-1, and LSF compared to the control was evident (Fig. 17A, compare lane 1 and 5; 17B, compare lane 2 and 5; 17C, compare lane 3 and 5). When nuclear extracts were probed for c-Myc, cells transfected with YY-1, LSF, and tat yielded the same amount of protein as the untransfected cells (Fig. 17A, compare lanes 2,3,4 with 5). Similarly, transfection with c-myc, LSF, and tat and immunoblotting with anti-YY-1 did not alter the expression patterns of YY-1 with respect to the control (Fig. 17B, compare lanes 1, 3, 4 with 5). The western data illustrated in Fig. 17D demonstrates that protein was equally loaded and verifies that c-Myc, YY-1 and LSF were in fact overexpressed.

5.6 The Effect of c-Myc, YY-1 and LSF on HIV-1 Virus Production

Cooperation in repression of HIV-1 LTR gene expression by c-Myc, YY-1, and LSF was demonstrated by co-transfection studies in Jurkat cells. Therefore, we wanted to investigate the effect of these factors on growth kinetics and virus production by transient co-transfection of the entire viral genome in target and non-target cell lines. Control transfections were performed with the unmodified vector(s). Culture supernatants were recovered 24, 48 and 72 hours post-transfection and analyzed for reverse transcriptase activity.

As 293-T cells support viral replication but cannot be infected, a measurement of the effects of c-Myc, YY-1 and LSF on a single round of replication can be made. The

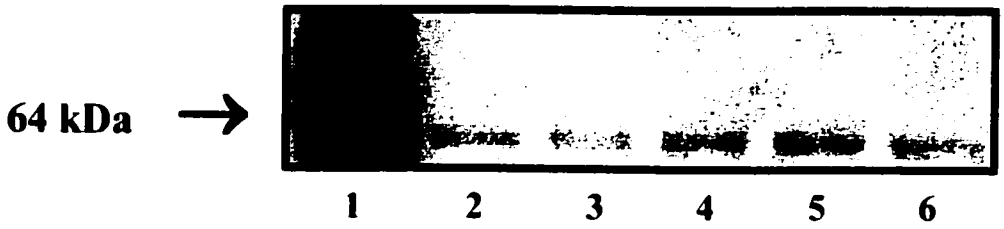
Figure 17: Western immunoblotting results in transfected 293-T cells

293-T cells were transfected with c-myc, YY-1, LSF, and tat as previously described. Cells were lysed to obtain nuclear extracts, separated by 10% SDS-PAGE and transferred to a sold support membrane. Western blots were probed for A) c-Myc, B) YY-1, C) LSF and D) B-actin expression. The lanes are represented as follows:

1) c-Myc (1.0 μg)
2) YY-1 (1.0 μg)
3) LSF (1.0 μg)
4) Tat (0.25 μg)
5) Tat (1.0 μg)
6) Mock transfected (control)

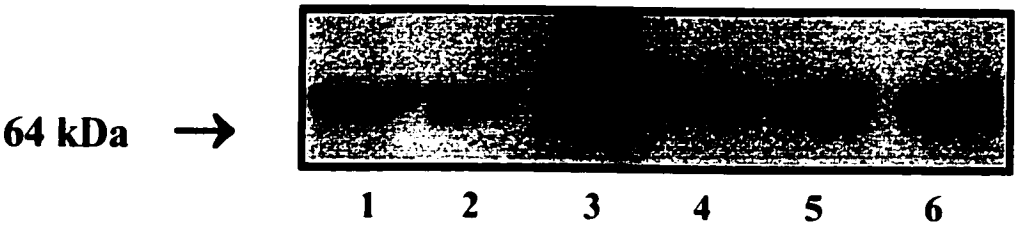
Western Blot Results in Transfected 293-T Cells

A) c-Myc

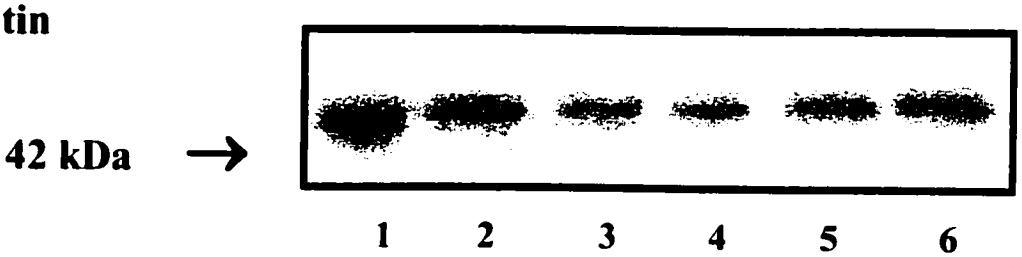


B) YY-1

C) LSF



D) B-actin



compiled results are shown graphically in Figure 18. YY-1 and LSF reduced virus production by 47.5% 72 hours post-transfection with respect to the unmodified vectors. These results are statistically significant for *P<0.05 and concurs with previous reports in HELA cells (96). Overexpression of c-Myc, YY-1, and LSF attenuated reverse transcriptase activity by 33.6%, while c-Myc alone inhibited HIV production by 10% relative to their respective controls. The results were not found to be statistically significant, although the observed pattern was consistent.

The data in Figure 19 demonstrate the average results obtained in Jurkat cells. C-Myc reduced virus production by approximately 10% after 72 hours in culture with respect to cells transfected with the control unmodified vector. Co-overexpression of YY-1 and LSF and c-Myc, YY-1, and LSF demonstrated 30.9% and 46% less virus, respectively. The results were only found to be statistically significant when all three transcription factors were overexpressed for *P<0.05. Thus, in CD4-expressing cells, the effects on virus production were more than additive amongst the cellular factors, c-Myc, YY-1, and LSF relative to the control.

The growth kinetics amongst the two cell types differed. In Jurkat cells, reverse transcriptase activity steadily increased with time, whereas in 293-T cells, virus production increased and then showed signs of leveling off by the third day in culture. This effect is likely attributable to multiple rounds of infection in Jurkat cells that were untransfected with the transcription factors.

Figure 18: The effect of c-Myc, YY-1 and LSF on HIV virus production in 293-T cells

293-T cells were transiently transfected with an HIV-1 infectious molecular clone, and 1.0 μg each of various combinations of transcription factors or the respective control unmodified vectors. Culture supernatants were recovered 24, 48 and 72 hours post-transfection and analyzed for reverse transcriptase activity. The results are expressed as ± SEM for n=3. The results for c-Myc and c-Myc/YY-1/LSF control co-transfections are shown as overlapping lines. Statistical significance was determined by one-way ANOVA, *$P<0.05$.

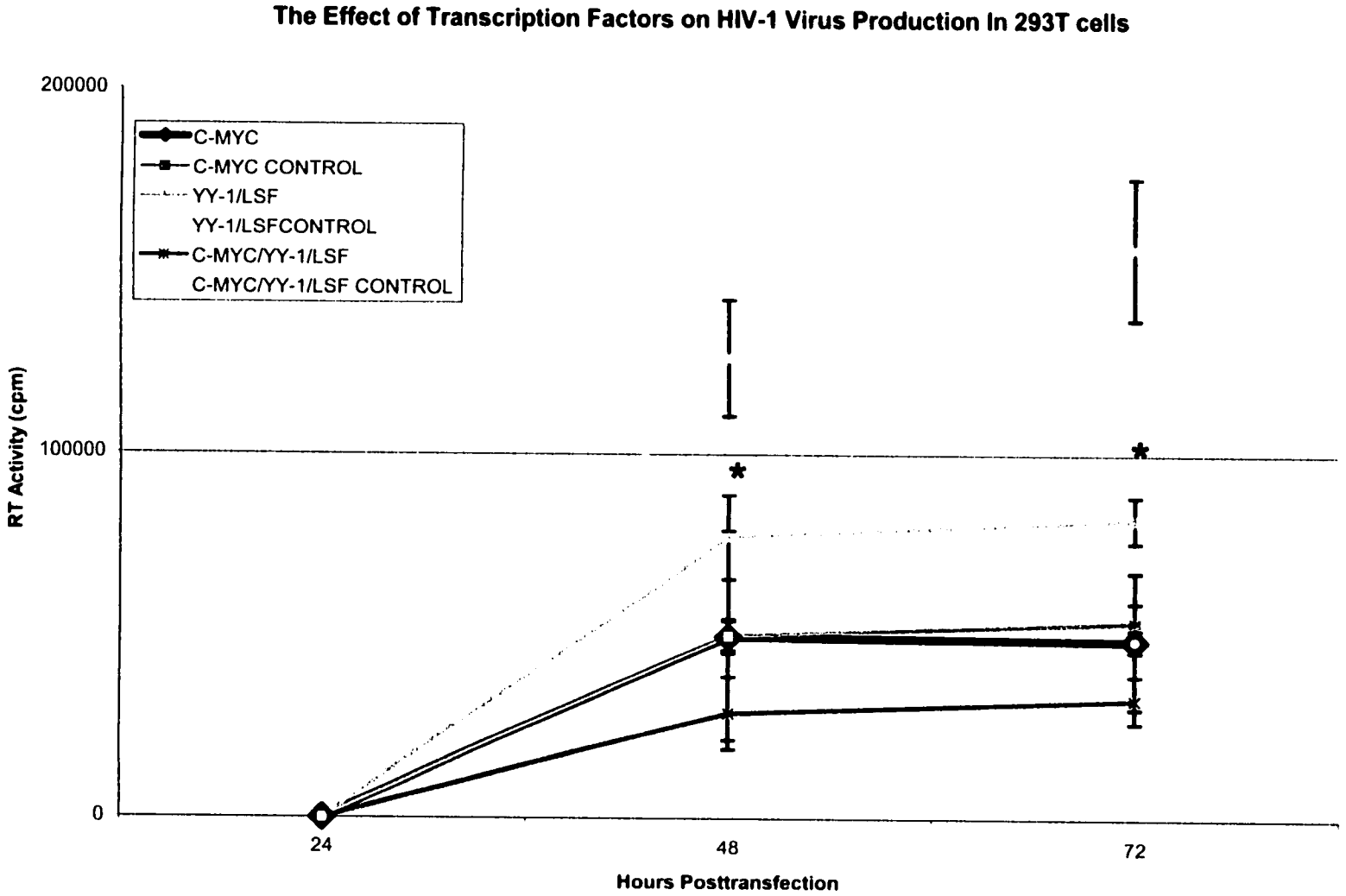
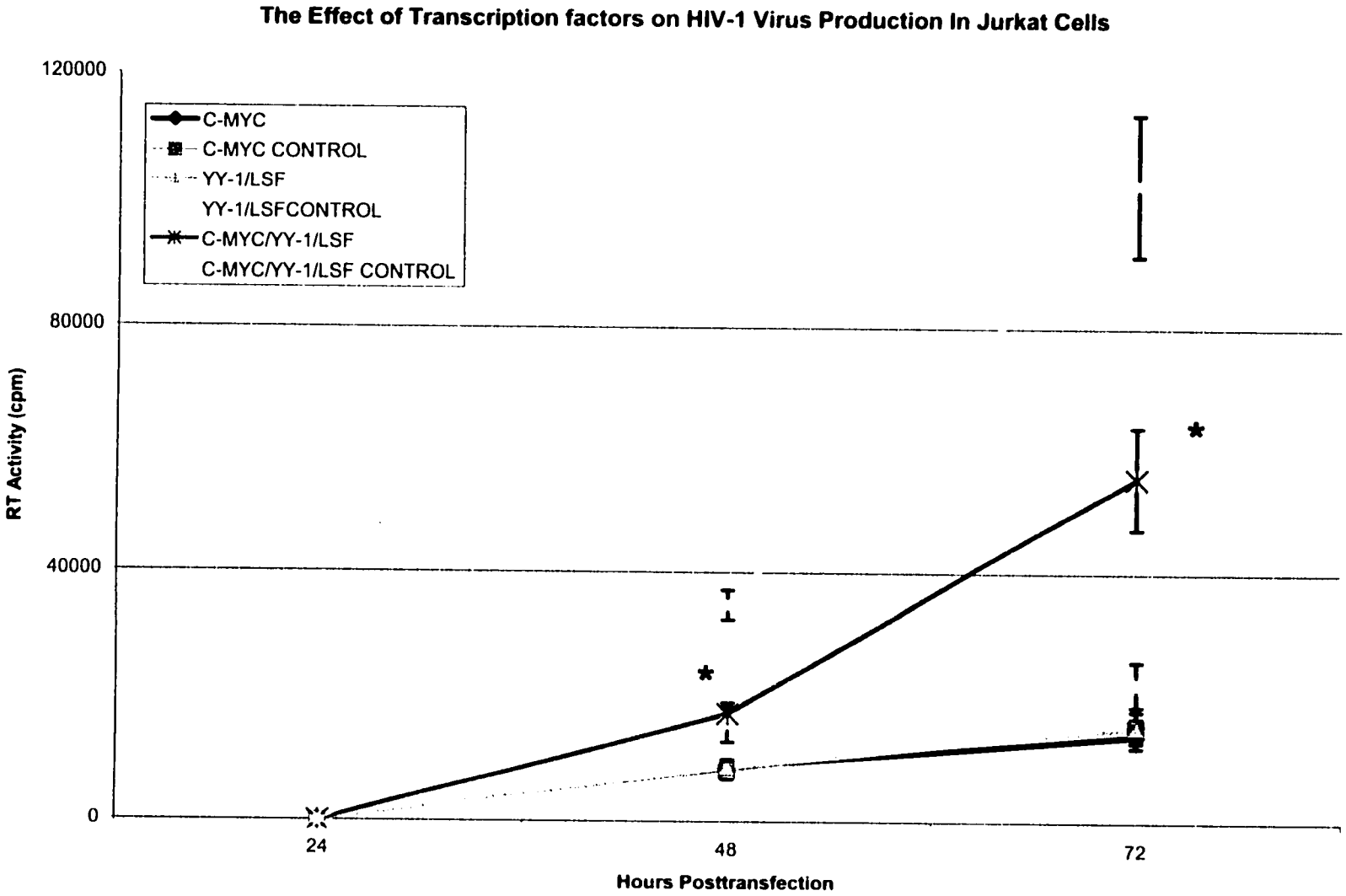

The Effect of Transcription Factors on HIV-1 Virus Production In 293T cells
200000
100000
0
RT Activity (cpm)
C-MYC
C-MYC CONTROL
YY-1/LSF
YY-1/LSFCONTROL
C-MYC/YY-1/LSF
C-MYC/YY-1/LSF CONTROL
*
*
24
48
72
Hours Posttransfection

Figure 19: The effect of c-myc, YY-1 and LSF on virus production in Jurkat cells

Jurkat cells were transiently transfected with an infectious molecular clone of HIV-1 and 1.0 μg each of various combinations of transcription factors or their control unmodified vectors. Culture supernatants were recovered from each sample 24, 48, and 72 hours post-transfection and analyzed for reverse transcriptase activity. The results are presented as a line graph expressed as ± SEM for n=3. The results for c-Myc, c-Myc control and YY-1/LSF co-transfections are shown as overlapping lines.
Statistical significance was determined by one-way ANOVA, *P<0.05.


The Effect of Transcription factors on HIV-1 Virus Production In Jurkat Cells
120000
80000
40000
0
RT Activity (cpm)
C-MYC
C-MYC CONTROL
YY-1/LSF
YY-1/LSFCONTROL
C-MYC/YY-1/LSF
C-MYC/YY-1/LSF CONTROL
24
48
72
Hours Posttransfection

5.7 c-Myc YY1 and LSF Co-Immunoprecipitate *in vivo*

It has been reported that c-myc and YY1 are capable of interacting in a manner that is dependent upon their relative levels (112). Furthermore, LSF and YY1 have been demonstrated to interact *in vivo* (20, 96). Therefore, we wanted to determine if endogenous levels of c-Myc could co-immunoprecipitate with YY1 and LSF in a human HIV target cell line. Jurkat CD4+ T-cell whole cell extracts were incubated with anti-YY1, anti-LSF, anti-c-Myc, or a nonspecific rabbit polyclonal antibody. Antibody-protein complexes were precipitated by the addition of anti-IgG protein A sepharose beads and centrifugation. The precipitates were then assayed for the presence of YY1, LSF, and c-Myc by Western blot analysis. The results are illustrated in figure 20.

YY1-specific complexes are detected in immunoprecipitation reactions of YY1, LSF and c-Myc (Fig. 20A). Similarly, c-Myc and YY1 could immunoprecipitate LSF (Fig. 20B). Quantitation of these results by densitometric analysis revealed that anti-YY1 antibodies could recover 52% of LSF in the cell and anti-c-Myc antibodies precipitated 75% of LSF that could be acquired by anti-LSF. Finally, a c-Myc specific band was evident in immunoprecipitates with YY1 and LSF (Fig. 20C). The results are specific because only trace amounts of protein were recovered by the nonspecific polyclonal antibody and the mock immunoprecipitation. This indicates that endogenous levels of c-Myc, YY1 and LSF are capable of interacting *in vivo* in the absence of the HIV-1 LTR. Furthermore, it provides suggestive evidence that a tertiary complex comprised of c-Myc, YY1 and LSF pre-assembles in the cell and is recruited to the HIV-1 LTR. The exact nature of these interactions warrants further investigation.

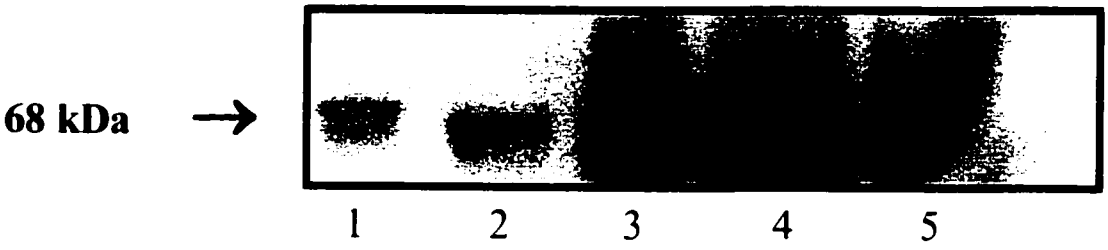
Figure 20: **c-Myc, YY1 and LSF interact *in vivo***

Whole cell extracts (WCE) were prepared from Jurkat cells and immunoprecipitated with anti-c-myc, anti-YY1,anti-LSF, irrelevant rabbit IgG antibodies, or left untreated. The complexes were captured using protein A sepharose beads, boiled and the supernatants were loaded on a 10% SDS-polyacrylamide gel. The results are shown as immunoblots probing for A) YY-1 expression, B) LSF expression and C) c-Myc expression. The lanes are defined as follows:

1) WCE alone (No immunoprecipitating antibody included)
2) WCE immunoprecipitated with nonspecific rabbit IgG antibody
3) WCE immunoprecipitated with anti-YY1
4) WCE immunoprecipitated with anti-LSF
5) WCE immunoprecipitated with anti-c-Myc

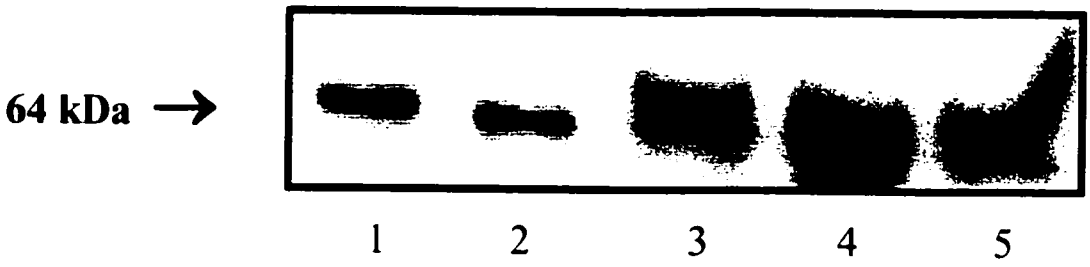
Co-Immunoprecipitation of c-Myc, YY-1 and LSF

A) YY-1 Western



B) LSF Western

C) c-Myc Western



5.8 Electrophoretic mobility shift complexes formed by Jurkat nuclear extracts

c-Myc facilitated repression of promoter activity has been previously demonstrated to occur through initiator elements (62,79). Since we observed cooperative inhibition with YY1 and LSF in transient co-transfections, we sought evidence that c-Myc could recognize and bind the HIV-1 LTR.

A population of Jurkat cells was transiently transfected as previously discussed. Nuclear extracts were treated with [γ-^{32}P]-end-labeled probe corresponding to the -19 to +26 region of the HIV-1 promoter which includes known binding sites for YY-1, LSF, and USF. Non-specific binding was reduced by the addition of carrier DNA (poly(dI-dC)). In competition gel shift studies, nuclear extracts were pre-incubated with a 100-fold excess of unlabeled probe. In antibody depletion experiments, the binding reactions were supplemented with a nonspecific antibody, pre-immune serum, or a mouse monoclonal anti-c-myc antibody. Complexes were resolved by non-denaturing polyacrylamide gel electrophoresis, and visualized by autoradiography. The results are captured in Figure 21.

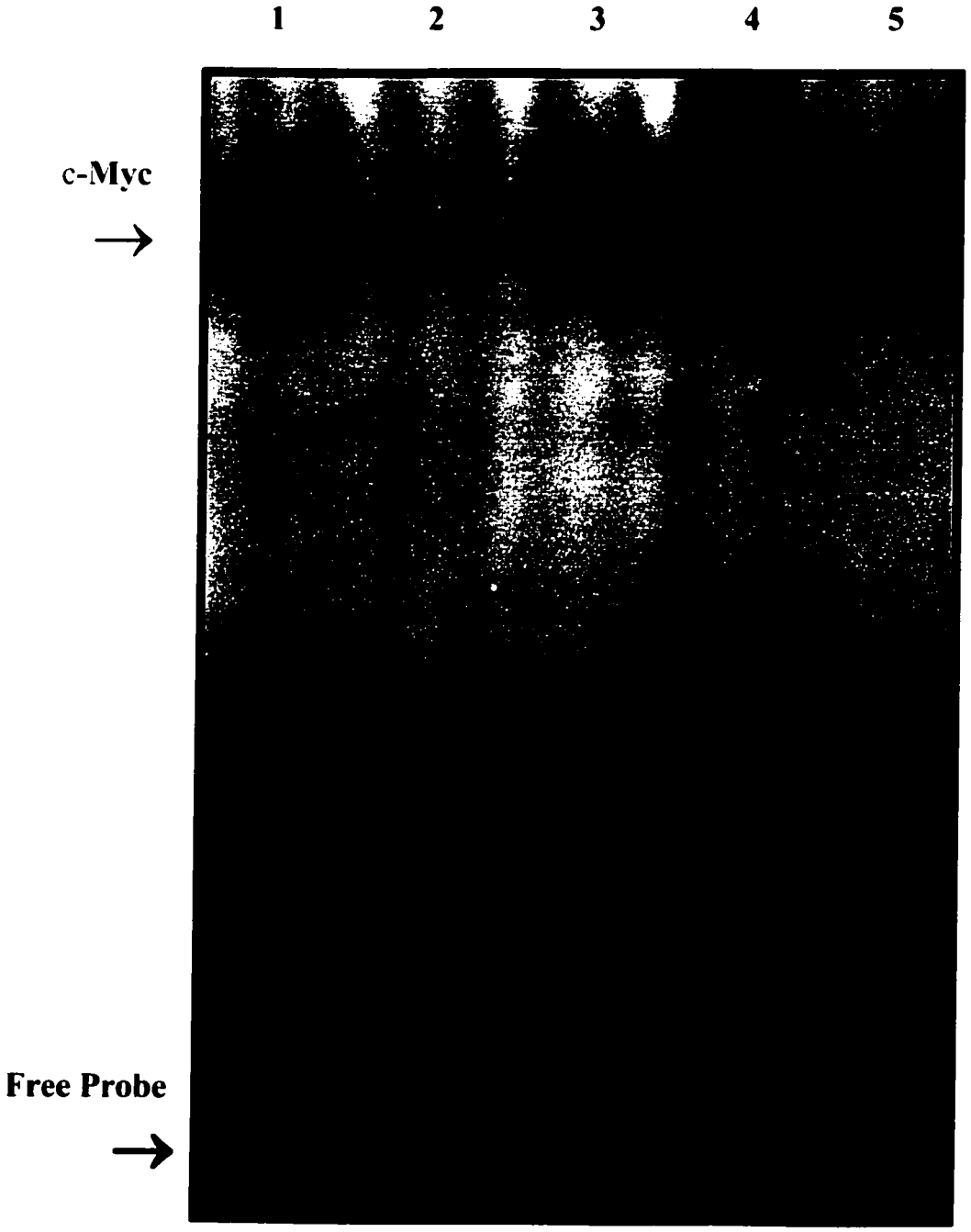
The effect of c-Myc antibody on electrophoretic mobility shift complexes formed with the LTR oligonucleotide provided evidence that c-Myc is involved in complex formation (Figure 21, compare lane 1 with lane 5). Pre-immune serum or irrelevant antibodies (anti-GST) had no effect on complex formation (Figure 21, compare lane 1 with lanes 3 and 4). The competition reaction (Figure 21, lane 2) demonstrates that complexes formed were sequence specific since they could be abrogated by the inclusion of the unlabeled probe.

Figure 21: Autoradiograph of electrophoretic mobility shift complexes formed by Jurkat cell nuclear extracts

The c-Myc specific complex is abrogated in the presence of anti-c-Myc antibody.
Cells were transfected as previously described. Nuclear extracts were probed with an oligonucleotide encoding the HIV-1 LTR from -19 to +26 with respect to the transcriptional start site.
The lanes are represented as follows:

1) C-Myc (1.0 μg)
2) Competition
3) C-Myc (1.0 μg) + nonspecific antibody (2.0 μg)
4) C-Myc (1.0 μg) + pre-immune serum (2.0 μg)
5) C-Myc (1.0 μg) + anti-c-Myc antibody (2.0 μg)

The Electrophoretic Mobility Shift Assay



5.9 Characterization of the HIV-1 LTR domain(s) targeted in c-Myc-mediated repression

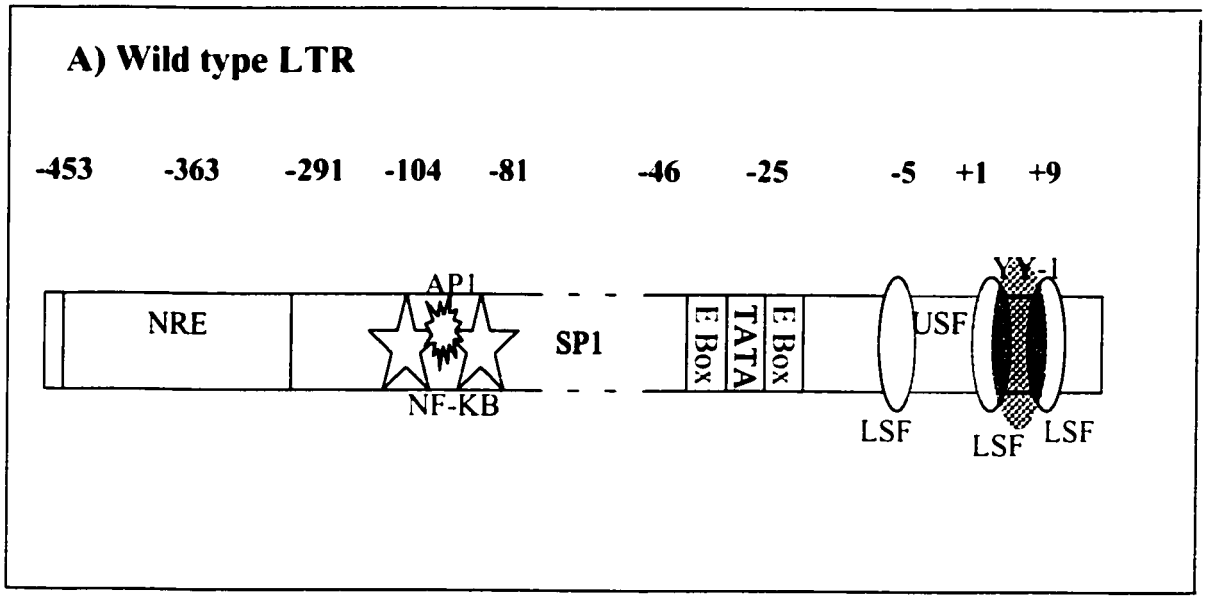
In an effort to elucidate the mechanism(s) of c-Myc mediated repression, we embarked on structure-function studies of the HIV-1 LTR In principle, c-myc-induced repression should be reversed once the region that is targeted on the HIV-1 LTR is deleted. Jurkat and 293-T cells were transiently co-transfected with linker scanner mutants lacking the upstream half (LS -21/-4) and the downstream half (LS -3/+15) of the LTR initiator linked to the CAT reporter in the presence of tat and/or 1.0 μg of c-Myc and/or 1.0 μg each of c-Myc, YY-1 and LSF. Figure 22 presents a schematic diagram of the structure of the wild type HIV-1 LTR and the linker scanner mutants used in these experiments.

The data in Figure 23 depicts the average results obtained in Jurkat cells. The repressive effect of c-Myc was relieved when the –21 to -4 region was removed, but not when the sequences corresponding to –3 to +15 were replaced. Expression from LS –3 to +15 in the presence of c-Myc was 44.3% ± 0.59 relative to the control. This result was statistically significant, *$P<0.001$. This was less than c-Myc-mediated repression typically observed with the wild type HIV-1 LTR (69% ± 2.96) and suggests the presence of a weak positive factor that binds to the –3 to +15 region and diminishes the ability for c-Myc to repress when it is exerting its effect at the –21 to –4 area. Cells that received c-Myc, YY-1 and LSF increased gene expression to 173% ± 1.667 and 171% ± 0.833 for LS –3/+15 and LS –21/-4 mutants, respectively. These results were found to be significant, **$P<0.001$. This was not unexpected since the YY-1 and LSF binding sites are located within the initiator element. However, expression was approximately 71% to

Figure 22: Schematic representation of the structure of the HIV-1 LTR

The structure of the wild type LTR including relevant binding proteins and their respective positions is shown in A.
The linker scanner mutants that were tested encompass the initiator element and are shown in part B. The white box defines the 18 base pair region that was replaced with the restriction enzyme sequence for NdeI-XhoI-SalI and the nucleotides with respect to the transcriptional start site are also indicated.

Schematic Representation of the HIV-1 LTR and Linker Scanner Mutants



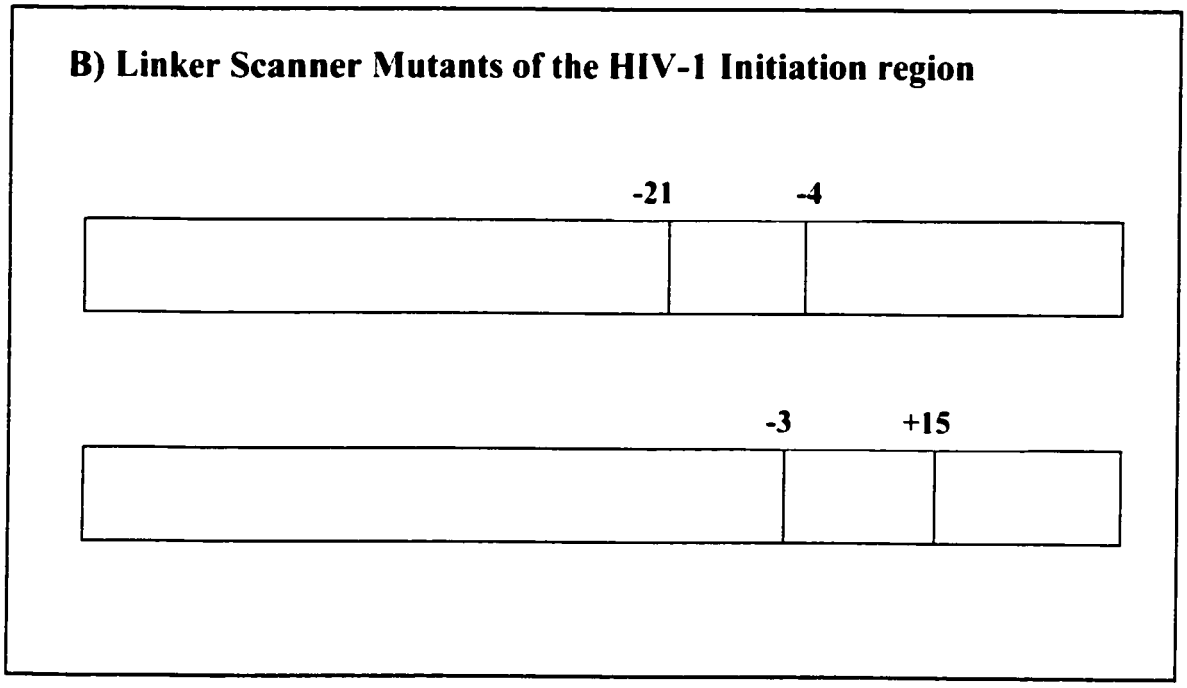
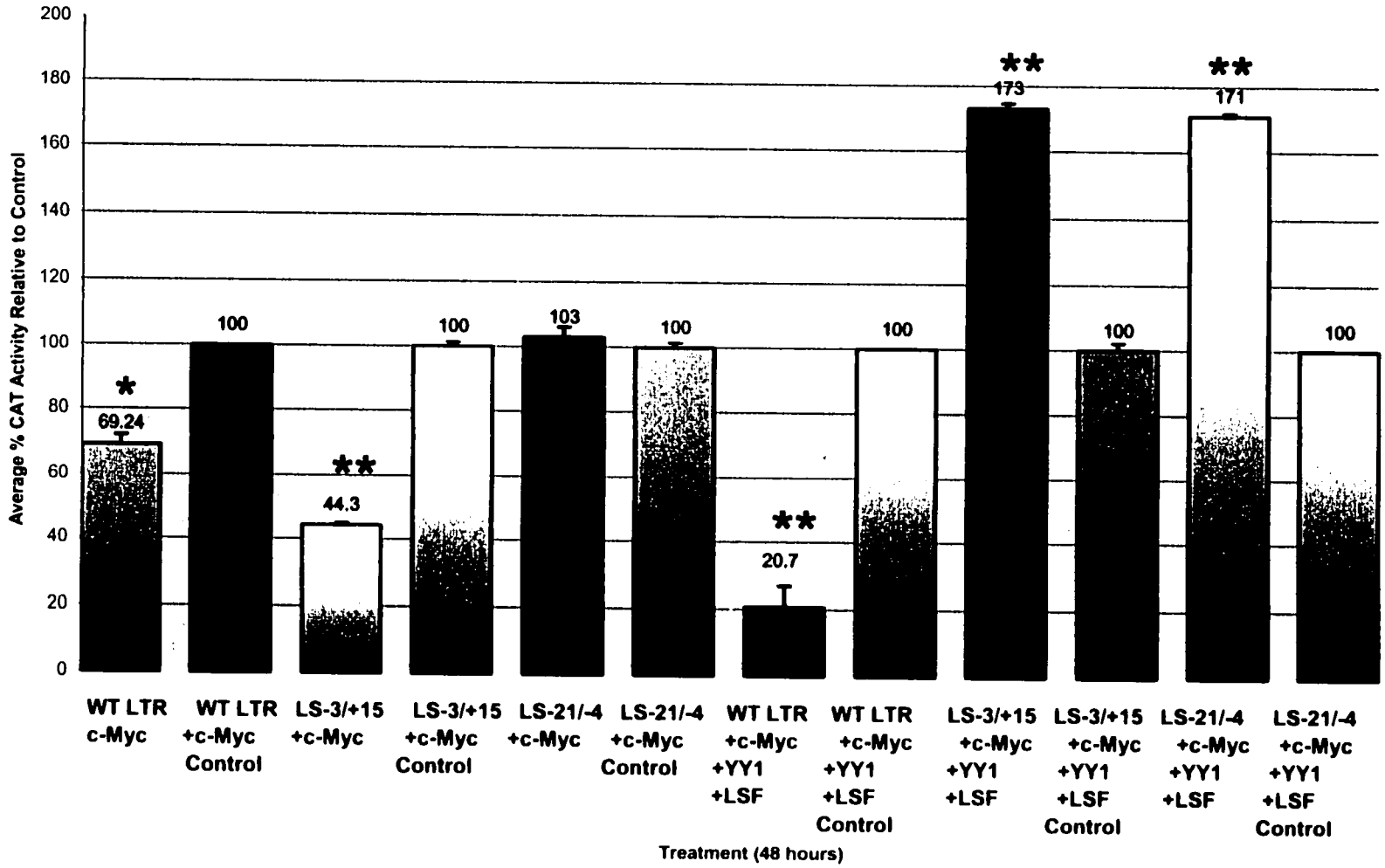


Figure 23 : Characterization of the HIV-1 LTR domain(s) involved in c-Myc-mediated repression in Jurkat cells

Jurkat cells were transiently transfected with linker scanner mutants linked to the CAT reporter, tat, and c-myc or c-myc, YY-1 and LSF as previously described. Control experiments were performed with the linker scanner, tat, and the appropriate unmodified vectors. The results are presented graphically as the average percent CAT activity relative to control and are expressed as +SEM for n=3. Statistical significance was determined by the one-way ANOVA, *P<0.05, **P<0.001.

Characterization of the c-Myc Target Domain of the HIV-1 LTR by Linker Scanner Mutagenesis In Jurkat Cells



73% greater in the presence of the transcription factors compared to the control which suggests that they are being titrated to other sites on the LTR where they can exert a positive regulatory effect. This idea is not unprecedented since there is evidence that USF stimulates when it binds to the initiator, yet represses when it binds to an E box element more upstream on the HIV-1 LTR (22).

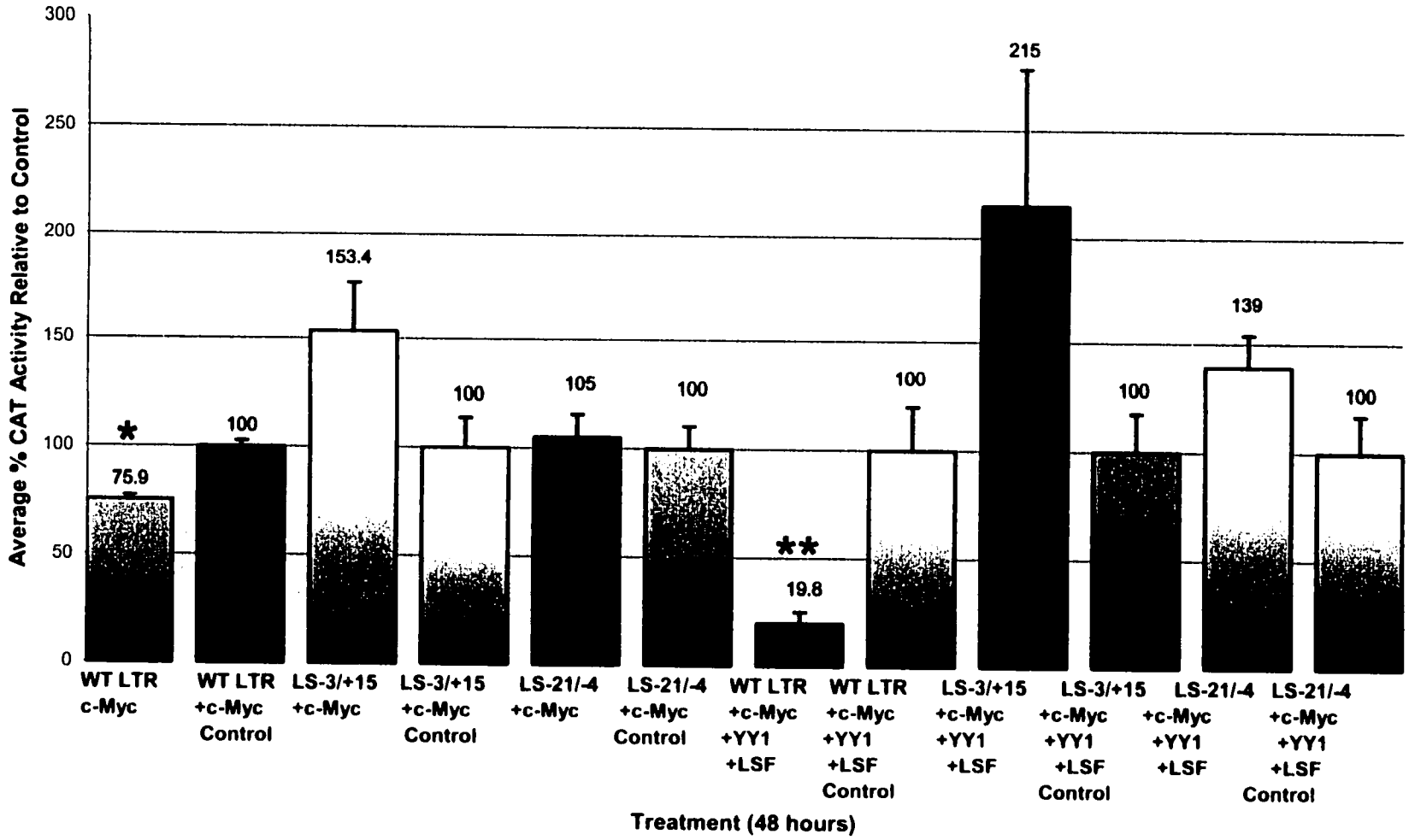
The data shown in Figure 24 describes the average results of the effect of c-myc on linker scanner mutants of the HIV-1 LTR in 293-T cells. C-myc repressed the wildtype HIV-1 LTR by 24.1% ± 2.0 relative to the unmodified vector control. These results were found to be statistically significant as determined by one-way ANOVA, *$P<0.05$. The c-myc effect was lost when both the –21 to –4 and –3 to +15 regions were removed. Expression from the LS –3/+15 in the presence of c-myc was 153.4% ± 23.4 and was not significantly different in comparison with the control. Similarly, expression from cells cotransfected with c-myc and LS –21/-4 was 105% ± 10.58 and was not statistically significant with respect to its control. This indicates that c-myc exerts its repressive effect through both upstream and downstream components of the initiator.

In agreement with the results from Jurkat cells, c-myc, YY-1 and LSF co-expression diminished wild type HIV-1 LTR-CAT activity by 80.2% ± 5.3 with respect to the control which was statistically significant, **$P<0.001$. Expression was heightened when cells were transfected with all three factors and LS –21/-4 or LS –3/+15 to 139% ± 15.6 and 215% ± 63.7, respectively, although the results were deemed not to be statistically significant by the one-way ANOVA test.

Figure 24 : Characterization of the HIV-1 LTR functional domains that mediate c-Myc repression in 293-T cells

Cells were transfected with either wild type HIV-1 LTR, or linker scanner mutants lacking nucleotides –21 to –4 and –3 to +15 with c-myc or c-myc, YY-1 and LSF. The results are represented graphically as the average percent CAT activity relative to the control and are expressed as +SEM for n=3. Statistical significance was deduced from the one-way ANOVA test, *P<0.05, **P<0.001.

Characterization of the c-Myc Target Domain on the HIV-1 LTR by Linker Scanner Mutagenesis in 293-T cells



5.10 Characterization of c-Myc Functional Domains

We sought to define the domain(s) of c-Myc that mediate repression of HIV-1 promoter activity by performing structure-function analyses with a series of c-myc deletion and point mutants spanning the amino terminus. A schematic representation of the structure of the wild type form of c-myc and the panel of deletion mutants examined can be found in Figure 25. Cells were transiently co-transfected with the wild type HIV-1 LTR-CAT reporter construct, tat and the mutants. Results were quantitated by phosphorimaging as the average percent CAT metabolism with respect to wild type c-myc.

The data presented in Figure 26 describe the effect of c-myc mutants on HIV-1 LTR-CAT expression in 293-T cells. Wild type c-myc reduced expression of the HIV-1 promoter by 49.4% ± 25.36 with respect to the unmodified control vector. The two partial MBI deletion mutants, d55-92 and d41-53 completely reversed HIV-1 LTR inhibition that was observed with wild type c-Myc. HIV-1 LTR-CAT expression was enhanced to 225.7% ± 41.63 and 189% ± 4.1 for d55-92 and d41-53 mutants, respectively. Consistent with our findings was the report that the loss of this region resulted in partial enhancement of the adenovirus major late promoter (62). The double phosphorylation mutant, T58A/S62A (threonine to alanine/serine to alanine) located within MBI also relieved HIV-1 LTR-CAT inhibition to the same extent. The results were found to be statistically significant as determined by the one-way ANOVA test, *$P<0.05$. While the T58A mutant did show increased gene expression over the wildtype (173% ± 54), it was not statistically significant. Taken together, the results indicate that

Figure 25: c-Myc protein structure

Schematic representation of wild type and deletion mutants of c-Myc used in the analysis for transregulatory domains involved in repression of HIV-1 promoter activity. The amino terminus spans amino acids 1 to 143 and includes the transactivation domain and two highly conserved elements, Myc Box I (MBI) and Myc box II (MBII). The carboxyl terminal domain is comprised of the basic region, BR (aa 355-367), and the helix-loop-helix, HLH (aa 368-410), leucine zipper, LZ (aa 411-439).
Phosphorylation sites are designated as **P.**
The panel of c-Myc mutants tested span the amino terminus and are illustrated directly below the wild type figure. Deletion mutants are described as a discontinuity in the diagram, while point mutants are designated by an **X.**

Mutations Spanning the Amino Terminal Transactivation Domain of the c-Myc Protein

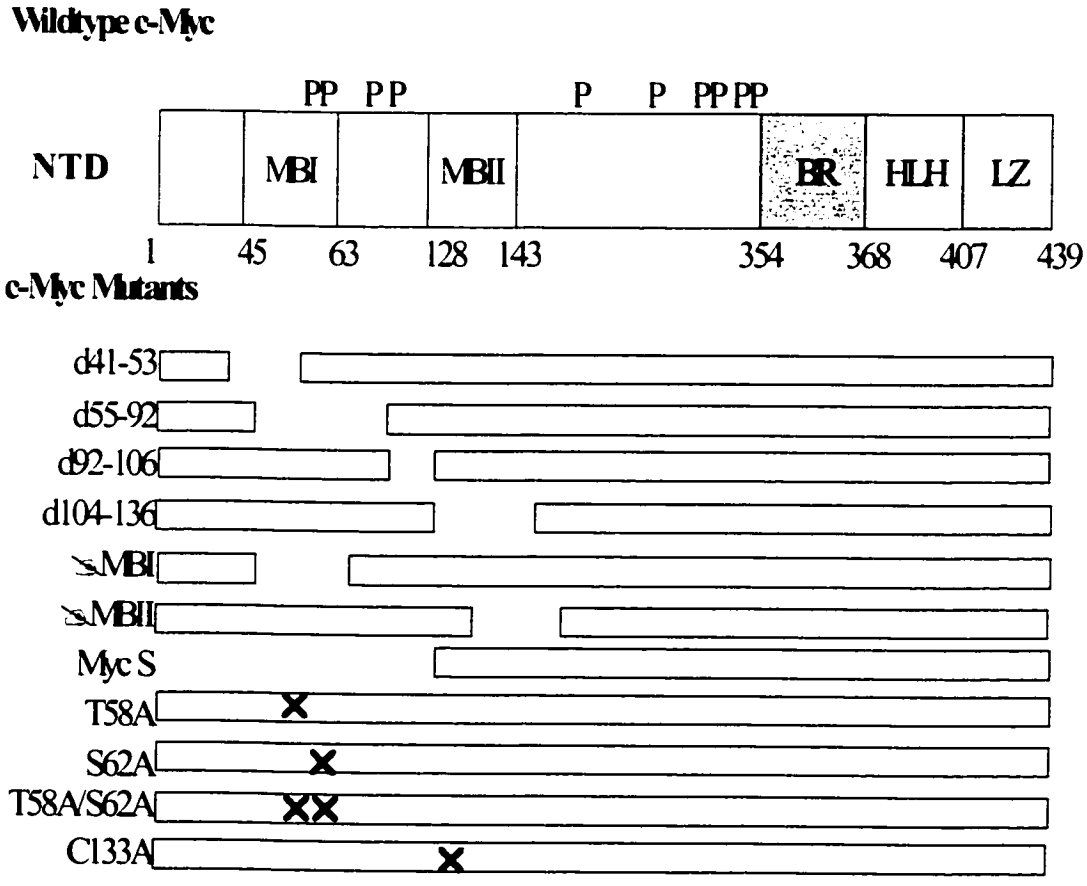
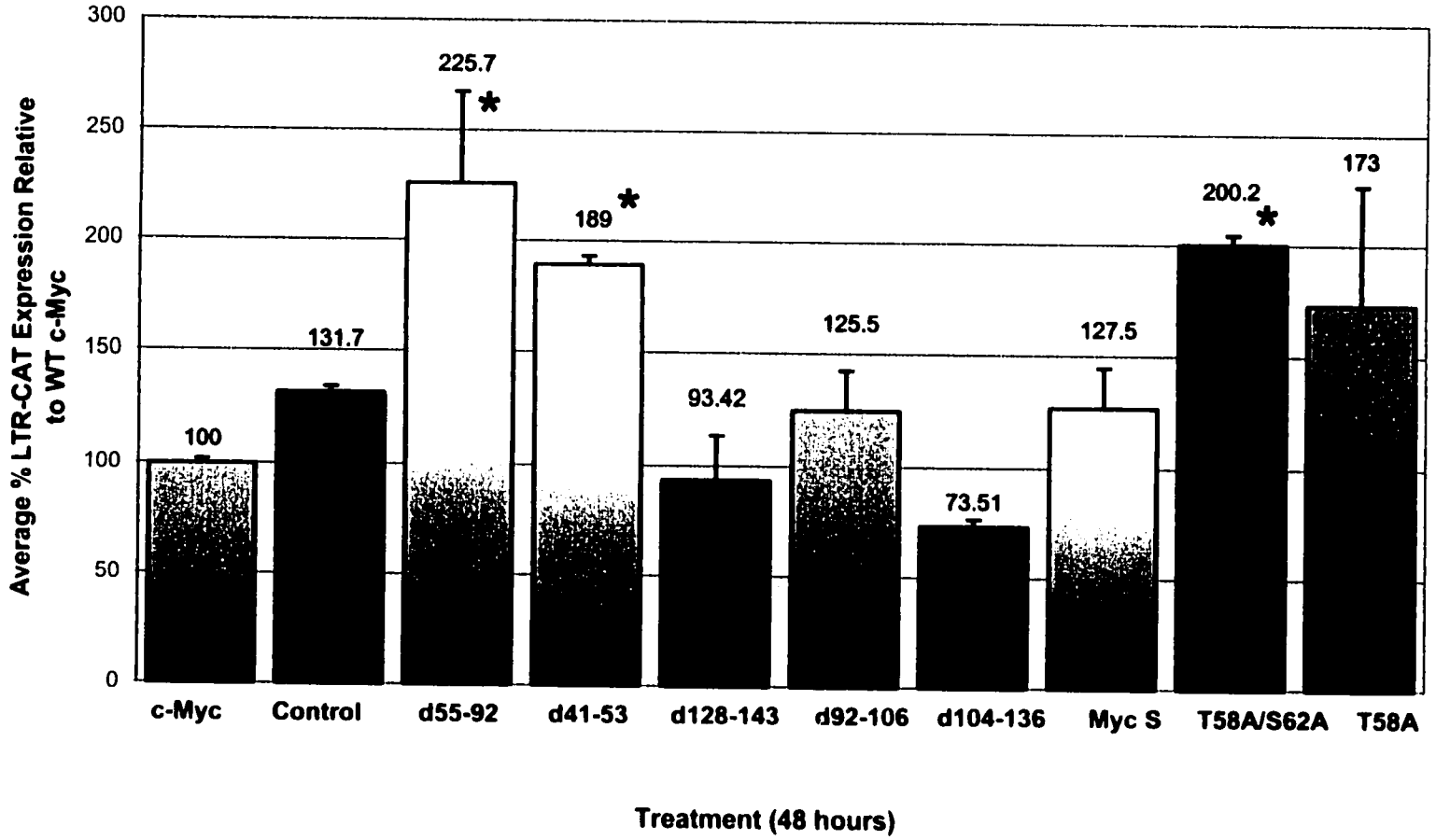


Figure 26: Characterization of the c-Myc functional domain(s) involved in repression of the HIV-1 LTR in 293-T cells

A series of c-myc mutants was transiently cotransfected with the HIV-1 LTR-CAT reporter in the presence of tat as previously described. The results are illustrated graphically as the average percent CAT activity with respect to wild type c-Myc and are expressed as +SEM, for n=3. Statistical significance was determined by one-way ANOVA, *$P<0.05$.

Characterization of the Functional Domains Involved in c-Myc-Facilitated Repression of the HIV-1 LTR in 293-T Cells



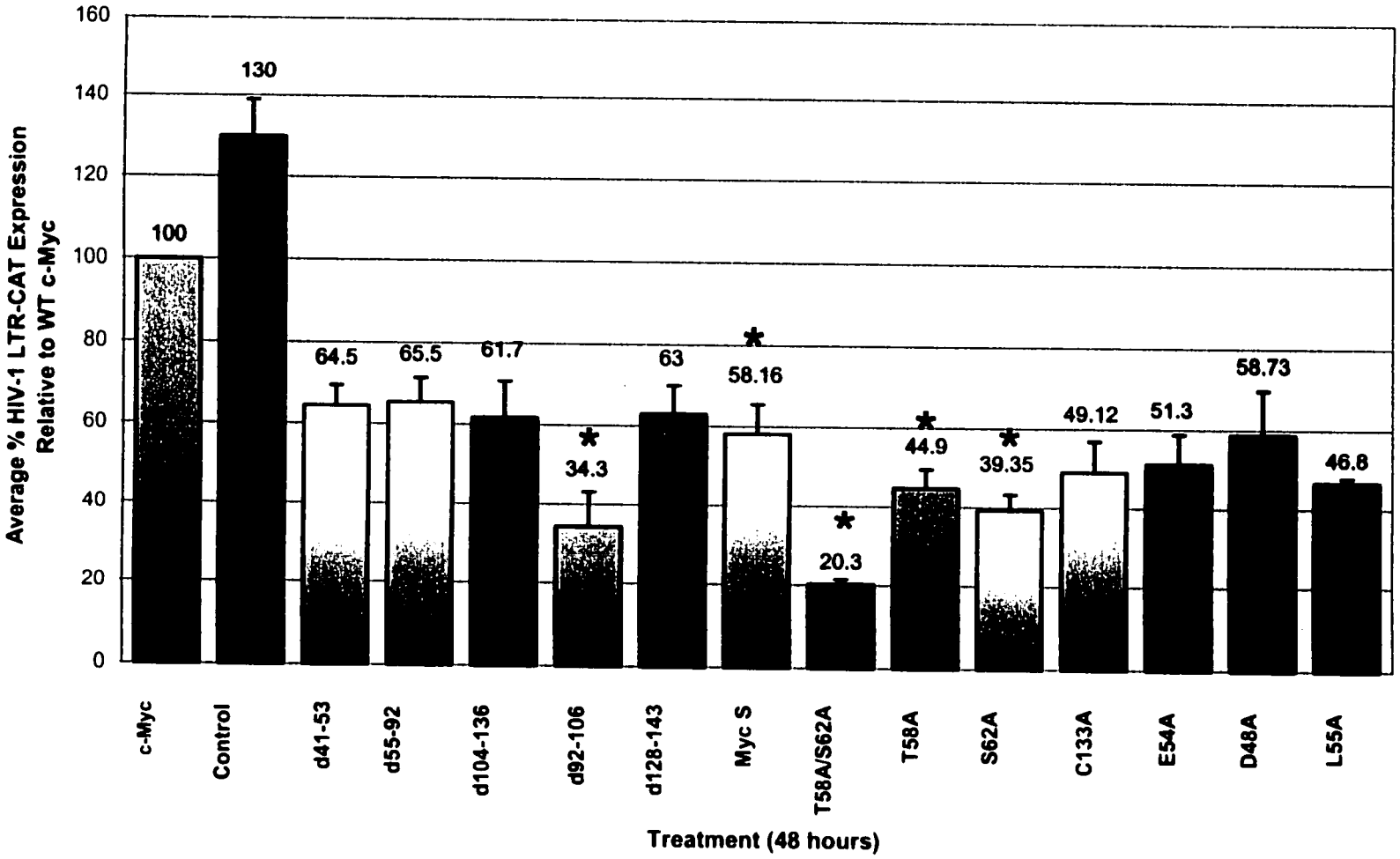
MBI and potentially the phosphorylation status of c-myc is important for repression of the HIV-1 promoter in 293-T cells. Indeed, it is known that mutations at T58A/S62A increase protein half-life which could exacerbate the transcriptional effects of c-myc at the target gene (79). The ΔMBII and d104-136 mutants partially increased gene expression, however, the results were not statistically different with respect to wild type c-myc. Myc S is a naturally occurring isoform that lacks the first 100 amino acids including MBI, but retains MBII. Thus, we would expect that this mutant would behave similarly to the MBI deletion mutants. However, no significant difference relative to wild type c-myc was observed. This suggests that the first 43 residues up to but not including MBI are involved in activation. The region represented by amino acids 92-106 appears to be dispensable since no significant effect was observed compared to wild type c-myc.

The data in Figure 27 shows the compiled quantitated results of c-myc mutagenesis studies performed in Jurkat cells. The panel of c-myc mutants assayed enhanced repression from the HIV-1 promoter by as little as 34.5% to as much as 79.3% with respect to the wild type form. These results indicate that residues 1 to 143 are involved in the transactivation function of c-myc and provide suggestive evidence that a more downstream site could be implicated in the repression effect. In this cell line, the MBI deletion mutants did not reverse myc-induced repression of HIV-1 LTR activity. Thus, it suggests that multiple and/or alternative mechanisms may be involved in different cell types perhaps attributable to relative amounts of c-myc protein or the presence or absence of host factors that exist in one cell line, but not the other.

Figure 27: Characterization of the c-myc functional domain(s) involved in repression of HIV-1 LTR expression

Jurkat cells were transiently transfected with the HIV-1 LTR-CAT reporter, tat, and the c-myc mutants. Results were analyzed by CAT assay and quantitated by phosphorImaging. The graph shows the average percent CAT activity relative to the wildtype form of c-Myc. The results are expressed as +SEM for n=5. Statistical significance was determined by one-way ANOVA, *$P<0.05$.

Characterization of the Functional Domains Involved in c-Myc-Mediated Repression of the HIV-1 LTR in Jurkat Cells



Discussion

In this study, it was demonstrated that the cellular transcription factor, c-myc down-regulates tat-mediated activation of HIV-1 LTR expression in a concentration-dependent manner. Transfection of 1.0ug to 2.0ug of c-myc reduced expression by approximately 31% and 43% with respect to the control. While the effect of c-myc on the HIV-1 LTR was relatively modest, these results are consistent with numerous reports describing only a 4- to 10-fold change in promoter expression (reviewed in reference 44). Cell viability studies confirmed that c-myc-induced repression is not due to toxicity as a result of transfection nor potentiation of apoptosis which frequently occurs as a consequence of c-myc overexpression (Caro, C., unpublished results). Furthermore, the effect cannot be due to squelching which arises when activators such as USF and YY-1 are overexpressed since this phenomenon was routinely detected with transfections that employed 10- to 20-fold greater amounts of DNA than were used in our studies (106,111).

YY-1 has been shown to physically associate with c-myc and the interaction between these proteins appears to result in reciprocal inhibition of their trans-regulatory functions (92,112-114). Thus, co-expression of c-myc and YY-1 was expected to reverse YY-1 mediated inhibition of the HIV-1 LTR. In contrast, the mutual effect of these transcription factors exerted on the HIV-1 promoter was approximately additive. The ability for c-myc and YY-1 to cooperate in function has not been previously described. Cooperative repression was also observed with co-expression of YY-1 and LSF, and c-myc and LSF. However, transfection with all three transcription factors reduced tat-

activated HIV-1 LTR expression by as much as 86%. The cooperative effects we observed at the HIV-1 promoter may be due to facilitation of alterations in the chromatin architecture by DNA remodeling enzymes, DNA bending, or the exclusion of positive acting factors with overlapping binding sites. One of the limitations of performing transient co-expression studies is that outcomes are detected on a naked DNA template. Consequently, it would be very informative to investigate the effect of these transcription factors by stable transfection experiments that reproduce the full complexity of gene regulation in the natural chromatin context. All things considered, the results suggest that c-myc, YY-1 and LSF could be members of a multiprotein complex although we cannot yet exclude the possibility that their combined effect on the LTR is due to the consolidation of multiple pathways.

Immunoblotting experiments were conducted to confirm that repression of the HIV-1 LTR was due to overexpression of the transcription factors. Significant overexpression in Jurkat cells was not observed. In contrast, the transcription factors were overexpressed in 293-T cells. These results are likely attributable to the low percentage of cells that are being transfected in Jurkat cells compared to 293-T cells and the differences in transfection methods employed. The results also indicate co-transfections of tat, c-myc, YY-1 and LSF do not regulate each others protein synthesis as differences were not detected in the expression patterns of any transcription factor with respect to the control. This implies that the effect of c-myc on the HIV-1 LTR is not indirect.

Co-immunoprecipitation studies were performed to determine if c-myc, YY-1 and LSF could interact with each other *in vivo* and in the absence of the HIV-1 LTR. Indeed, we confirmed previous reports that c-myc and YY-1 could associate (112). C-

Myc interacts with the glycine-alanine-rich (amino acids 154-198) and zinc finger (amino acids 295-414) functional domains of YY-1 (7,112,120). While YY-1 contacts the central region (amino acids 250-353) and basic-Helix-Loop-Helix motif (amino acids 345-439) of c-Myc (112). The central region of c-myc is highly conserved amongst species and members of the c-myc family and also appears to be functionally important in conferring YY-1 binding specificity. Antibodies raised against YY-1 could also immunoprecipitate LSF. Similarly, LSF antibodies could immunoprecipitate YY-1. The LSF region that interacts with YY-1 has been mapped to residues 164 to 403, while the YY-1 functional domains that associate with LSF are zinc fingers 1 and 2 (20). Given that the binding sites for LSF and c-Myc partially overlap on YY-1 suggests that their interaction with YY-1 is mutually exclusive. Alternatively, it is conceivable that all three proteins could form a complex with more tenuous interactions. It was reported that YY-1 and LSF could physically associate at the HIV-1 initiator element and inhibit transcription by a mechanism that involves YY-1-facilitated recruitment of histone deacetylase (20). Furthermore, the interaction of YY-1 and the co-repressor occurs at the central residues (170 to 200) of YY-1 to which c-Myc was shown to bind (112). This predicts that c-myc would compete for binding to YY-1 with histone deactylase (HDAC). However, it should be mentioned that there is conflicting evidence regarding the involvement of the YY-1 central domain in c-myc binding. While two research groups had identified the C-terminus (7,112), only one group reported the central region was involved (112). Thus, we cannot conclude explicitly that YY-1 simultaneously interacts with histone deacetylase, c-myc and LSF, although it remains a possibility. Given that LSF and c-myc can mutually co-immunoprecipitate each other indicates that YY-1 is not required to

bridge these factors in a multiprotein complex and therefore issues related to competition, and mutual exclusion are not necessarily pertinent. This result also coincides with the co-transfection experiments in which c-myc and LSF-mediated repression of the HIV-1 LTR was more than additive. Since LSF has not been previously identified as a c-myc-interacting protein, future studies examining the functional domains involved would greatly expedite a more thorough understanding of this potential multiprotein complex at the HIV-1 LTR. Experimentally, this could be achieved by performing GST pull-down assays with wild type and mutant forms of c-myc, and LSF. Nonetheless, the co-immunoprecipitation studies do lend clues into the assembly process of the putative c-myc,YY-1 and LSF network. Taking into account that the cells were not transfected with the HIV-1 LTR insinuates that if the transcription factors were to form a complex they would be capable of doing so prior to binding to the promoter. The functional significance of this multiprotein complex could be to reduce initiation, or elongation of the RNA polymerase by recruitment of co-repressors and chromatin remodeling enzymes or effectively create a "roadblock" that attenuates promoter clearance.

The effects of c-myc, YY-1 and LSF on viral production and growth kinetics were measured by testing the culture supernatants for reverse transcriptase activity in Jurkat and 293-T cell lines. As Jurkat cells are human CD4+ T-lymphocytes, they can support multiple rounds of infection which could compromise detection of the c-myc effect on the HIV-1 promoter, however they were chosen for our studies because they provide the most physiologically relevant conditions. To overcome this limitation, virus production was measured at multiple intervals over a brief length of time since transfections were transient. Indeed, while the effect of the transcription factors was evident after 72 hours,

reverse transcriptase activity was increasing. In contrast, 293-T cells cannot be re-infected with replicating virions and thus issues related to multiple rounds of infection are not applicable. In Jurkat cells, expression of c-myc, YY-1 and LSF additively reduced reverse transcriptase activity compared to the control. However, the effect of c-myc on virus production was less than could be predicted by the expression studies. This may be due to the effect of other viral factors which were absent in the LTR-CAT experiments thereby partially masking c-myc-mediated repression. Alternatively, c-myc may be exerting a positive effect elsewhere in the viral genome or at other phases of the life cycle. For example, ini1 is a nucleocytoplasmic protein that was demonstrated to interact with HIV integrase and facilitate preinitiation complex integration. C-Myc may play a role in this process since it is known to interact with ini1 (44,48,79). In support of this idea is the evidence that c-myc upregulates the CXCR4 promoter, the co-receptor for HIV entry. The mechanism involves an indirect effect of c-myc and relief of YY-1-mediated repression upon co-overexpression (74). Additionally, c-myc has been demonstrated to regulate nuclear import of HIV-1 DNA by regulating gene expression of cellular factors that control the nuclear import machinery (92).

It is noteworthy that independent investigations in our laboratory have yielded conflicting results. The effect of c-myc on virus production had been previously examined in Jurkat cells and quantitated by testing for the presence of p24 gag. The results obtained indicated a statistically significant reduction in virus production by approximately 30% relative to the control (Caro, C., unpublished results). The discrepancy may be due to differences in the method of transfection, or the relative sensitivity of the assays employed. Nonetheless, c-myc does appear to repress virus

production, although the extent of that effect remains in question. Moreover, the effect of c-myc on HIV-1 LTR-CAT expression has been clearly established, and the effects of c-myc, YY-1 and LSF on virus production are consistent amongst the two cell lines examined.

Since c-myc can interact with the initiator binding protein, YY-1 and has been demonstrated to repress through initiator elements (33,64,85,86,89,127), it was important to determine whether c-myc could recognize the HIV-1 initiator. The results of the electrophoretic mobility shift assay confirmed that c-myc formed a specific protein-DNA complex which could be abrogated in the presence of c-myc antibodies, but not preimmune serum or an irrelevant antibody. This result is consistent with previous reports that YY-1 and LSF can bind to this region (96) and supports our model of cooperation amongst the three factors. However, this assay does not lend insight into the exact nature of c-myc binding to this portion of the LTR. C-myc could be binding directly to DNA given that the initiator element also includes an E box motif or it could interact with YY-1, or LSF proteins which bind DNA through their respective sites. To further delineate the characteristics of c-myc interaction with the initiator, an *in vitro* band shift could be performed with purified factors added sequentially to the reaction mixture. If c-myc is recruited to the LTR by protein-protein interactions, then the absence of either YY-1 or LSF should preclude c-myc binding. This type of experiment would also show whether or not these transcription factors are sufficient for binding to the LTR probe and discern if co-factors are required. Alternatively, electrophoretic mobility shift assays using oligonucleotides mutated in the E box, YY-1, and LSF binding sites may also be able to differentiate direct binding of c-myc, versus protein-protein interactions.

However, it should be cautioned that the binding sites for these factors are located in very close proximity, and even overlap to some extent. Thus, mutation of adjacent sites could influence the binding specificity of proteins and procure misleading results. In addition, footprinting and/or chromatin immunoprecipitation studies would be beneficial to confirm the electrophoretic mobility shift experiments in an *in vivo* context that re-creates the natural chromatin structure.

The c-myc responsive site on the HIV-1 LTR was characterized by linker scanning mutagenesis in Jurkat and 293-T cell lines. On the basis of the results obtained from the electrophoretic mobility shift, and co-immunoprecipitation studies, we narrowed our scope to the initiation region. Transient transfections were performed with two mutants lacking either the upstream or downstream portion of the HIV-1 initiator in the presence or absence of c-myc, YY-1 and LSF. In principle, if a domain was involved in c-myc-mediated repression of the LTR, we would expect a reversal in the expression pattern relative to the wild type promoter. In Jurkat cells, c-myc lost the ability to repress in the absence of the –21/-4 region which corresponds to a portion of the low affinity binding site for LSF and the proximal E box flanking the TATA motif. However, it retained the ability to repress HIV-1 LTR expression when co-transfected with the linker scanner mutant lacking the –3/+15 domain which includes two high affinity binding sites for LSF and the USF E box. Unfortunately, the precise location of the YY-1 binding site has not been finely characterized, and therefore it is difficult to conclude if the c-myc effect was relieved because it interacts with YY-1, the low affinity LSF binding site, or the proximal E box. When cells were transfected with c-myc, YY-1 and LSF, expression was enhanced beyond the control. These results are consistent with the cooperative

network model and provide suggestive evidence that the transcription factors are being titrated to other regions of the LTR where they can exert a positive regulatory effect. The hypothesis is supported by investigations that identified differential effects of USF on the HIV-1 LTR. It was found that when USF binds to an E box motif in the negative regulatory element, it reduces transcription, whereas binding to the initiation region stimulated transcription (22). To date, however, additional YY-1 and LSF binding sites have not been characterized. In 293-T cells, c-myc repression was observed to approximately the same extent as in Jurkat cells. While linker scanning mutagenesis did implicate the initiation region, repression was reversed for all of the mutants tested and co-overexpression of c-myc, YY-1 and LSF heightened expression from the LTR mutants in agreement with the pattern observed in Jurkat cells. Since the complete length of the initiation region was important for c-myc mediated repression in 293-T cells, but the –21/-4 domain was sufficient in Jurkat cells suggests that alternative or additional mechanisms could be involved in different cell lines or cell types.

Since most USF binding sites can also be recognized by other HLH proteins, it was thought that c-myc could outcompete USF for binding to the E box and reverse stimulation. This hypothesis was tested by transient co-transfections with increasing amounts of c-myc. In Jurkat and 293-T cells, c-myc was not able to interfere with USF mediated stimulation. This result provides further support in favor of c-myc interacting with YY-1 and LSF rather than direct DNA binding. These results are not necessarily unexpected given that the E box motif is not the ideal consensus sequence and is consistent with previous reports that the affinity for USF binding to E box elements is higher than c-myc.

While we have focused our attention on the HIV-1 initiation region, we do not ignore the possibility that c-myc may act either directly or indirectly at other sites along the U3 and R regions. For example, c-myc may repress gene expression by sequestering Sp1 which binds to nucleotides -453 to –416 and –75 to –40 on the HIV-1 LTR as it has been demonstrated to do at the p21 promoter (42). In addition, previous reports have demonstrated that c-myc can down-regulate expression of CCAAT transcription factor/nuclear factor 1 dependent promoters by suppressing the activity of this protein (64). Indeed, this motif is found downstream of the first initiator element on the HIV-1 LTR at nucleotides +40 to +46 with respect to the start site of transcription. It is intriguing that the CCAAT sequence overlaps the second HIV-1 initiator element and TAR RNA. This raises the prospect that c-myc could regulate transcription at both initiation regions which is consistent with the dose-dependent inhibition we observed. Furthermore, it would be worthwhile to investigate the effect of c-myc on tat and TAR in the future since TAR RNA overlaps both initiators. Also, c-myc has been shown to bind to the PTEFb elongation complex and transactivate (30,79) in a manner analogous to tat. This suggests that c-myc may compete for binding to PTEFb with tat. The net effect would be a reduction in gene expression not owing to active repression, but weaker transactivation. This hypothesis could be tested by performing primer extension experiments to determine if the processivity of the RNA polymerase is reduced or not in the presence of c-myc versus tat.

To better appreciate c-myc's role at the HIV-1 LTR, we sought to identify the c-myc domain involved in transcriptional repression by performing structure-function studies with deletion mutants spanning the amino terminus. Although investigations with

the adenovirus major-late promoter determined that MBII and to a lesser extent MBI was involved in repression (62), mutants of these sites did not abolish or attenuate expression from the HIV-1 LTR in Jurkat cells. Indeed, the panel of c-myc mutants tested enhanced CAT activity indicating that the role of the amino terminus in the context of the HIV-1 promoter is to facilitate transactivation. It would be intriguing to perform similar studies in the future with c-myc mutants corresponding to the YY-1 and LSF sites to explicitly determine if repression of the HIV-1 LTR occurs via protein-protein interactions.

In contrast, mutants of the MBI domain and in particular the serine-62/threonine-58 phosphorylation residues increased gene expression in 293-T cells relative to the wild type form of c-myc. A number of proteins have been shown to bind to this region of c-myc and regulate its biological activity including TBP (104). Given that both c-myc and YY-1 can bind TBP is consistent with the possibility that c-myc sequesters TBP and prevents productive interactions between TBP and YY-1 resulting in inhibition of HIV-1 LTR expression. Alternatively, another candidate protein is Bin1 which was originally identified in a two-hybrid screen with MBI domain as the bait (104). Bin1 is a nucleocytoplasmic adapter protein that is in excess to c-myc in cells and functions by inhibiting the oncogenic and transactivation properties of c-myc. However, Bin1 regulation of c-myc activity may not be the mechanism involved in LTR repression because its binding also depends on MBII which was not demonstrated to play a role in our studies.

The discrepancy amongst the two cell lines in detecting the c-myc repression domain may be due to the presence or absence of other factors or the relative amounts of endogenous protein present. Western immunoblotting results demonstrated that the

amount of c-myc in Jurkat cells was greater than 293-T cells. As a result, the background levels could mask the effect of the deletion mutants in Jurkat cells. In an effort to overcome this limitation, various cell lines were tested for their c-myc levels. However, c-myc is an essential protein and is known to be present in low amounts only in resting and undifferentiated cells. Given that c-myc levels are strongly correlated to the cell cycle, it was thought that serum-starving cells prior to transfection would equilibrate the cells and down-regulate endogenous c-myc levels. However, c-myc is an early gene product that is rapidly up-regulated and is maximal within two to four hours following serum stimulation (93). Furthermore, the health of the cells was compromised and the stress of the transfection resulted in excessive amounts of death quashing the performance of subsequent reporter assays. A rat c-myc nullizygous cell line does exist, however it was not beneficial for use in these particular experiments because of their protracted doubling time and untransfectability.

While many questions remain unanswered and numerous possible mechanisms warrant further investigation, we have clearly shown that c-myc down-regulates expression of the HIV-1 LTR and virus production (albeit to a lesser extent). The effect appears to be facilitated by cooperation with the transcription factors, YY-1 and LSF at the initiator element overlapping the start site of transcription and not by direct binding to noncanonical E box motifs, or competition with USF. In terms of the clinical impact, it seems that c-myc could be an important factor in initiating and perpetuating the proviral latent state by facilitating HIV entry (74), nuclear import (92), and transcriptional repression.

Reference List

1. 1994. Retroviruses, p. 531-562. *In* D. O. White and F. J. Fenner (ed.), Medical Virology. Academic Press.
2. 1997. Failures of Host Defense Mechanisms, p. 10.22-10.36. *In* C. A. Janeway and P. Travers (ed.), Immunobiology: The Immune System in Health and Disease. Current Biology LTD/Garland Pub Inc, London.
3. **Amati, B., S. R. Frank, D. Donjerkovic, and S. Taubert**. 2001. Function of the c-Myc oncoprotein in chromatin remodeling and transcription. Biochim.Biophys.Acta **1471**:M135-M145.
4. **An, G., V. Hidakak, and L. Siminovitch**. 1982. Expression of bacterial B-galactosidase in animal cells. Mol.Cell Biol. **2**:1628-1632.
5. **Antoni, B. A., S. B. Stein, and A. B. Rabson**. 1994. Regulation of human immunodeficiency virus infection: implications for pathogenesis. Adv.Virus Res. **43**:53-145.
6. **Austen, M., B. Luscher, and J. M. Luscher-Firzlaff**. 1997. Characterization of the transcriptional regulator YY1. The bipartite transactivation domain is independent of interaction with the TATA box-binding protein, transcription factor IIB, TAFII55, or cAMP-responsive element-binding protein (CPB)-binding protein. J.Biol.Chem. **272**:1709-1717.
7. **Austen, M., C. Cerni, J. M. Luscher-Firzlaff, and B. Luscher**. 1998. YY1 can inhibit c-Myc function through a mechanism requiring DNA binding of YY1 but neither its transactivation domain nor direct interaction with c-Myc. Oncogene **17**:511-520.

8. **Bjorklund, S., G. Almouzni, I. Davidson, K. P. Nightingale, and K. Weiss**. 1999. Global transcription regulators of eukaryotes. Cell **96**:759-767.

9. **Blackwell, T. K., L. Kretzner, E. M. Blackwood, R. N. Eisenman, and H. Weintraub**. 1990. Sequence-specific DNA binding by the c-Myc protein. Science **250**:1149-1151.

10. **Blankson, J. N., D. Persaud, and R. F. Siliciano**. 2002. The challenge of viral reservoirs in HIV-1 infection. Annu.Rev.Med. **53**:557-593.

11. **Boyd, K. E., J. Wells, J. Gutman, S. M. Bartley, and P. J. Farnham**. 1998. c-Myc target gene specificity is determined by a post-DNAbinding mechanism. Proc.Natl.Acad.Sci.U.S.A **95**:13887-13892.

12. **Buratowski, S.** 1994. The basics of basal transcription by RNA polymerase II. Cell **77**:1-3.

13. **Bushmeyer, S., K. Park, and M. L. Atchison**. 1995. Characterization of functional domains within the multifunctional transcription factor, YY1. J.Biol.Chem. **270**:30213-30220.

14. **Butera S.T**. 2000. Therapeutic targeting of human immunodeficiency virus type-1 latency: current clinical realities and future scientific possibilities. Antiviral Research **48**:143-176.

15. **Carhew, R. W., L. A. Chodish, and P. A. Sharp**. 1985. An RNA polymerase II transcription factor binds to an upstream element in the adenoassociated virus major late promoter. Cell **43**:439-448.

16. **Chan, D. C. and P. S. Kim**. 1998. HIV entry and its inhibition. Cell **93**:681-684.

17. **Chun, R. F., O. J. Semmes, C. Neuveut, and K. T. Jeang**. 1998. Modulation of Sp1 phosphorylation by human immunodeficiency virus type 1 Tat. J.Virol. **72**:2615-2629.

18. **Claassen, G. F. and S. R. Hann**. 1999. Myc-mediated transformation: the repression connection. Oncogene **18**:2925-2933.

19. **Condra, J. H., M. D. Miller, D. J. Hazuda, and E. A. Emini**. 2002. Potential new therapies for the treatment of HIV-1 infection. Annu.Rev.Med. **53**:541-555.

20. **Coull J.J., Romerio F., Sun J-M., Volker J.L., Galvin K.M., Davie J.R., Shi Y., Hansen U., and Margolis D.M.** 2000. The Human Factors YY1 and LSF Repress the Human Immunodeficiency Virus Type 1 Long Terminal Repeat via Recruitment of Histone Deacetylase 1. Journal of Virology **74**:6790-6799.

21. **Cullen, B. R.** 1991. Regulation of human immunodeficiency virus replication. Annu.Rev.Microbiol. **45**:219-250.

22. **d'Adda, d. F., G. Marzio, M. I. Gutierrez, L. Y. Kang, A. Falaschi, and M. Giacca**. 1995. Molecular and functional interactions of transcription factor USF with the long terminal repeat of human immunodeficiency virus type 1. J.Virol. **69**:2765-2775.

23. **Dang, C. V.** 1999. c-Myc target genes involved in cell growth, apoptosis, and metabolism. Mol.Cell Biol. **19**:1-11.

24. **Demarchi, F., P. D'Agaro, A. Falaschi, and M. Giacca**. 1992. Probing protein-DNA interactions at the long terminal repeat of human immunodeficiency virus type 1 by in vivo footprinting. J.Virol. **66**:2514-2518.

25. **Demarchi, F., P. D'Agaro, A. Falaschi, and M. Giacca**. 1993. In vivo footprinting analysis of constitutive and inducible protein-DNA interactions at the long terminal repeat of human immunodeficiency virus type 1. J.Virol. **67**:7450-7460.

26. **Desbarats, L., S. Gaubatz, and M. Eilers**. 1996. Discrimination between different E-box-binding proteins at an endogenous target gene of c-myc. Genes Dev. **10**:447-460.

27. **Di Fagagna, F. D., G. Marzio, M. I. Gutierrez, L. Y. Kang, A. Falaschi, and M. Giacca**. 1995. Molecular and functional interactions of transcription factor USF with the long terminal repeat of human immunodeficiency virus type 1. J.Virol. **69**:2765-2775.

28. **Du, H., A. L. Roy, and R. G. Roeder**. 1993. Human transcription factor USF stimulates transcription through the initiator elements of the HIV-1 and the Ad-ML promoters. EMBO J. **12**:501-511.

29. **Eberhardy, S. R., C. A. D'Cunha, and P. J. Farnham**. 2000. Direct examination of histone acetylation on Myc target genes using chromatin immunoprecipitation. J.Biol.Chem. **275**:33798-33805.

30. **Eberhardy, S. R. and P. J. Farnham**. 2001. c-Myc mediates activation of the cad promoter via a post-RNA polymerase II recruitment mechanism. J.Biol.Chem. **276**:48562-48571.

31. **Eilers, M., D. Picard, K. R. Yamamoto, and J. M. Bishop**. 1989. Chimaeras of myc oncoprotein and steroid receptors cause hormone- dependent transformation of cells. Nature **340**:66-68.

32. **Emerman, M. and M. H. Malim**. 1998. HIV-1 regulatory/accessory genes: keys to unraveling viral and host cell biology. Science **280**:1880-1884.

33. **Facchini, L. M., S. Chen, W. W. Marhin, J. N. Lear, and L. Z. Penn**. 1997. The Myc negative autoregulation mechanism requires Myc-Max association and involves the c-myc P2 minimal promoter. Mol.Cell Biol. **17**:100-114.

34. **Facchini, L. M. and L. Z. Penn**. 1998. The molecular role of Myc in growth and transformation: recent discoveries lead to new insights. FASEB J. **12**:633-651.

35. **Freed, E. O.** 1998. HIV-1 gag proteins: diverse functions in the virus life cycle. Virology **251**:1-15.

36. **Fry, C. J. and P. J. Farnham**. 1999. Context-dependent transcriptional regulation. J.Biol.Chem. **274**:29583-29586.

37. **Furlong, E. E., T. Rein, and F. Martin**. 1996. YY1 and NF1 both activate the human p53 promoter by alternatively binding to a composite element, and YY1 and E1A cooperate to amplify p53 promoter activity. Mol.Cell Biol. **16**:5933-5945.

38. **Galvin, K. M. and Y. Shi**. 1997. Multiple mechanisms of transcriptional repression by YY1. Mol.Cell Biol. **17**:3723-3732.

39. **Gandhi, R. T. and B. D. Walker**. 2002. Immunologic control of HIV-1. Annu.Rev.Med. **53**:149-172.

40. **Garcia, J. A., F. K. Wu, R. Mitsuyasu, and R. B. Gaynor**. 1987. Interactions of cellular proteins involved in the transcriptional regulation of the human immunodeficiency virus. EMBO J. **6**:3761-3770.

41. **Garner, M. M. and A. Revzin**. 1981. A gel electrophoresis method for quantifying the binding of proteins to specific DNA regions: application to components of the E.coli lactose operon regulatory system. Nucleic Acids Res. **9**:3047-3060.

42. **Gartel, A. L., X. Ye, E. Goufman, P. Shianov, N. Hay, F. Najmabadi, and A. L. Tyner**. 2001. Myc represses the p21(WAF1/CIP1) promoter and interacts with Sp1/Sp3. Proc.Natl.Acad.Sci.U.S.A **98**:4510-4515.

43. **Gorman, C. M., L. F. Moffat, and B. H. Howard**. 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol.Cell Biol. **2**:1044-1051.

44. **Grandori, C., S. M. Cowley, L. P. James, and R. N. Eisenman**. 2000. The Myc/Max/Mad network and the transcriptional control of cell behavior. Annu.Rev.Cell Dev.Biol. **16**:653-699.

45. **Greenberg, M. E. and M. B. Mathews**. 1997. Effects of heterologous downstream sequences on the activity of the HIV- 1 promoter and its response to Tat. Nucleic Acids Res. **25**:5017-5024.

46. **Gregor, P. D., M. Sawadogo, and R. G. Roeder**. 1990. The adenovirus major late transcription factor USF is a member of the helix-loop-helix group of regulatory proteins and binds to DNA as a dimer. Genes Dev. **4**:1730-1740.

47. **Hueber, A. O., M. Zornig, D. Lyon, T. Suda, S. Nagata, and G. I. Evan**. 1997. Requirement for the CD95 receptor-ligand pathway in c-Myc-induced apoptosis [see comments]. Science **278**:1305-1309.

48. Jeang, K. T. HIV and cellular Factors. Virology , IV-1-IV-6. 96. Bethesda,MD.

Ref Type: Report

49. Jeang, K. T. HIV-1 Tat: Structure and Function. III-3-III-12. 1996. Bethesda, MD.

Ref Type: Report

50. **Jeang, K. T., H. Xiao, and E. A. Rich**. 1999. Multifaceted activities of the HIV-1 transactivator of transcription, Tat. J.Biol.Chem. **274**:28837-28840.

51. **Johnson, W. E. and R. C. Desrosiers**. 2002. Viral persistance: HIV's strategies of immune system evasion. Annu.Rev.Med. **53**:499-518.

52. **Jones, K. A., P. A. Luciw, and N. Duchange**. 1988. Structural arrangements of transcription control domains within the 5'-untranslated leader regions of the HIV-1 and HIV-2 promoters. Genes Dev. **2**:1101-1114.

53. **Kamp, W., M. B. Berk, C. J. Visser, and H. S. Nottet**. 2000. Mechanisms of HIV-1 to escape from the host immune surveillance. Eur.J.Clin.Invest **30**:740-746.

54. **Kiermaier, A., J. M. Gawn, L. Desbarats, R. Saffrich, W. Ansorge, P. J. Farrell, M. Eilers, and G. Packham**. 1999. DNA binding of USF is required for specific E-box dependent gene activation in vivo. Oncogene **18**:7200-7211.

55. **Kim, J. and D. J. Shapiro**. 1996. In simple synthetic promoters YY1-induced DNA bending is important in transcription activation and repression. Nucleic Acids Res. **24**:4341-4348.

56. **Kingsman, S. M. and A. J. Kingsman**. 1996. The regulation of human immunodeficiency virus type-1 gene expression. Eur.J.Biochem. **240**:491-507.

57. **Kretzner, L., E. M. Blackwood, and R. N. Eisenman**. 1992. Myc and Max proteins possess distinct transcriptional activities. Nature **359**:426-429.

58. **Lee, J. S., K. M. Galvin, and Y. Shi**. 1993. Evidence for physical interaction between the zinc-finger transcription factors YY1 and Sp1. Proc.Natl.Acad.Sci.U.S.A **90**:6145-6149.

59. **Lee, M. H., K. Sano, F. E. Morles, and D. T. Imagawa**. 1987. Sensitive RT assay to detect and quantitate HIV. Clinical Microbiology **25**:1717-1721.

60. **Lee, T. C. and E. B. Ziff**. 1999. Mxi1 is a repressor of the c-Myc promoter and reverses activation by USF. J.Biol.Chem. **274**:595-606.

61. **Levy, J. A.** 1993. Pathogenesis of human immunodeficiency virus infection. Microbiol.Rev. **57**:183-289.

62. **Li, L. H., C. Nerlov, G. Prendergast, D. MacGregor, and E. B. Ziff**. 1994. c-Myc represses transcription in vivo by a novel mechanism dependent on the initiator element and Myc box II. EMBO J. **13**:4070-4079.

63. **Li, X., Y. Quan, and M. A. Wainberg**. 1997. Controlling elements in replication of the human immunodeficiency virus type 1. Cell Mol.Biol.(Noisy.-le-grand) **43**:443-454.

64. **Lucas, J. M., N. M. Wilkie, and J. C. Lang**. 1993. c-MYC repression of promoter activity through core promoter elements. Biochem.Biophys.Res.Commun. **194**:1446-1452.

65. **Luscher, B. and L. G. Larsson**. 1999. The basic region/helix-loop-helix/leucine zipper domain of Myc proto-oncoproteins: function and regulation. Oncogene **18**:2955-2966.

66. **Maheswaran, S., H. Lee, and G. E. Sonenshein**. 1994. Intracellular association of the protein product of the c-myc oncogene with the TATA-binding protein. Mol.Cell Biol. **14**:1147-1152.

67. **Margolis, D. M., J. M. Ostrove, and S. E. Straus**. 1993. HSV-1 activation of HIV-1 transcription is augmented by a cellular protein that binds near the initiator element. Virology **192**:370-374.

68. **Margolis, D. M., M. Somasundaran, and M. R. Green**. 1994. Human transcription factor YY1 represses human immunodeficiency virus type 1 transcription and virion production. J.Virol. **68**:905-910.

69. **Marhin, W. W., S. Chen, L. M. Facchini, A. J. Fornace, Jr., and L. Z. Penn**. 1997. Myc represses the growth arrest gene gadd45. Oncogene **14**:2825-2834.

70. **Marzio, G. and M. Giacca**. 1999. Chromatin control of HIV-1 gene expression. Genetica **106**:125-130.

71. **McCune, J. M.** 1995. Viral latency in HIV disease. Cell **82**:183-188.

72. **McMichael, A.** 2002. T Cell Responses and Viral Escape. Cell **93**:673-676.

73. **Mitchell, P. J. and R. Tjian**. 1989. Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. Science **245**:371-378.

74. **Moriuchi, M., H. Moriuchi, D. M. Margolis, and A. S. Fauci**. 1999. USF c-Myc enhances, while Yin-Yang 1 suppresses, the promoter activity of CXCR4, a coreceptor for HIV-1 entry. J.Immunol. **162**:5986-5992.

75. **Naghavi, M. H., M. C. Estable, S. Schwartz, R. G. Roeder, and A. Vahlne**. 2001. Upstream stimulating factor affects human immunodeficiency virus type 1 (HIV-1) long terminal repeat-directed transcription in a cell-specific manner,

independently of the HIV-1 subtype and the core-negative regulatory element. J.Gen.Virol. **82**:547-559.

76. **Nathanson, N. and B. J. Mathieson**. 2000. Biological considerations in the development of a human immunodeficiency virus vaccine. J.Infect.Dis. **182**:579-589.

77. **Obaya, A. J., M. K. Mateyak, and J. M. Sedivy**. 1999. Mysterious liaisons: the relationship between c-Myc and the cell cycle. Oncogene **18**:2934-2941.

78. **Oster, S. K., W. W. Marhin, C. Asker, L. M. Facchini, P. A. Dion, K. Funa, M. Post, J. M. Sedivy, and L. Z. Penn**. 2000. Myc is an essential negative regulator of platelet-derived growth factor beta receptor expression. Mol.Cell Biol. **20**:6768-6778.

79. **Oster, S. K., C. S. Ho, E. L. Soucie, and L. Z. Penn**. 2002. The myc oncogene: MarvelouslY Complex. Adv.Cancer Res. **84**:81-154.

80. **Ou, S. H., L. F. Garcia-Martinez, E. J. Paulssen, and R. B. Gaynor**. 1994. Role of flanking E box motifs in human immunodeficiency virus type 1 TATA element function. J.Virol. **68**:7188-7199.

81. **Parada, C. A., J. B. Yoon, and R. G. Roeder**. 1995. A novel LBP-1-mediated restriction of HIV-1 transcription at the level of elongation in vitro. J.Biol.Chem. **270**:2274-2283.

82. Paul, W. E. Can the Immune Response Control HIV Infection?
Ref Type: Generic

83. **Paul, W. E.** 1997. Can the immune response control HIV infection? Cell177-182.

84. **Pazin, M. J. and J. T. Kadonaga**. 1997. What's up and down with histone deacetylation and transcription? Cell **89**:325-328.

85. **Peeters, A., P. F. Lambert, and N. J. Deacon**. 1996. A fourth Sp1 site in the human immunodeficiency virus type 1 long terminal repeat is essential for negative-sense transcription. J.Virol. **70**:6665-6672.

86. **Penn, L. J., M. W. Brooks, E. M. Laufer, T. D. Littlewood, J. P. Morgenstern, G. I. Evan, W. M. Lee, and H. Land**. 1990. Domains of human c-myc protein required for autosuppression and cooperation with ras oncogenes are overlapping. Mol.Cell Biol. **10**:4961-4966.

87. **Penn, L. J., M. W. Brooks, E. M. Laufer, and H. Land**. 1990. Negative autoregulation of c-myc transcription. EMBO J. **9**:1113-1121.

88. **Pereira, L. A., K. Bentley, A. Peeters, M. J. Churchill, and N. J. Deacon**. 2000. A compilation of cellular transcription factor interactions with the HIV-1 LTR promoter. Nucleic Acids Res. **28**:663-668.

89. **Peukert, K., P. Staller, A. Schneider, G. Carmichael, F. Hanel, and M. Eilers**. 1997. An alternative pathway for gene regulation by Myc. EMBO J. **16**:5672-5686.

90. **Philipp, A., A. Schneider, I. Vasrik, K. Finke, Y. Xiong, D. Beach, K. Alitalo, and M. Eilers**. 1994. Repression of cyclin D1: a novel function of MYC. Mol.Cell Biol. **14**:4032-4043.

91. **Prendergast, G. C.** 1999. Mechanisms of apoptosis by c-Myc. Oncogene **18**:2967-2987.

92. **Qyang, Y., X. Luo, T. Lu, P. M. Ismail, D. Krylov, C. Vinson, and M. Sawadogo**. 1999. Cell-type-dependent activity of the ubiquitous transcription factor USF in cellular proliferation and transcriptional activation. Mol.Cell Biol. **19**:1508-1517.

93. **Riggs, K. J., S. Saleque, K. K. Wong, K. T. Merrell, J. S. Lee, Y. Shi, and K. Calame**. 1993. Yin-yang 1 activates the c-myc promoter. Mol.Cell Biol. **13**:7487-7495.

94. **Rittner, K., M. J. Churcher, M. J. Gait, and J. Karn**. 1995. The human immunodeficiency virus long terminal repeat includes a specialised initiator element which is required for Tat-responsive transcription. J.Mol.Biol. **248**:562-580.

95. **Roeder, R. G.** 1996. The role of general initiation fators in transcription by RNA polymerase II. Trends Biochem.Sci. **21**:327-335.

96. **Romano, G., M. Kasten, G. De Falco, P. Micheli, K. Khalili, and A. Giordano**. 1999. Regulatory functions of Cdk9 and of cyclin T1 in HIV tat transactivation pathway gene expression. J.Cell Biochem. **75**:357-368.

97. **Romerio, F., M. N. Gabriel, and D. M. Margolis**. 1997. Repression of human immunodeficiency virus type 1 through the novel cooperation of human factors YY1 and LSF [published erratum appears in J Virol 1998 Feb;72(2):1709]. J.Virol. **71**:9375-9382.

98. **Rosen, C. A., J. G. Sodroski, and W. A. Haseltine**. 1985. The location of cis-acting regulatory sequences in the human T cell lymphotropic virus type III (HTLV-III/LAV) long terminal repeat. Cell **41**:813-823.

99. **Rosen, C. A.** 1991. Regulation of HIV gene expression by RNA-protein interactions. Trends in Genetics 7:9-14.

100. **Rosen, C. A.** 1992. HIV regulatory proteins: potential targets for therapeutic intervention. AIDS Res.Hum.Retroviruses **8**:175-181.

101. **Roulston, A., R. Lin, P. Beauparlant, M. A. Wainberg, and J. Hiscott**. 1995. Regulation of human immunodeficiency virus type 1 and cytokine gene expression in myeloid cells by NF-kappa B/Rel transcription factors. Microbiol.Rev. **59**:481-505.

102. **Rowland-Jones, S., S. Pinheiro, and R. Kaul**. 2001. New insights into host factors in HIV-1 pathogenesis. Cell **104**:473-476.

103. **Roy, A. L., M. Meisterernst, P. Pognonec, and R. G. Roeder**. 1991. Cooperative interaction of an initiator-binding transcription initiation factor and the helix-loop-helix activator USF. Nature **354**:245-248.

104. **Roy, A. L., C. Carruthers, T. Gutjahr, and R. G. Roeder**. 1993. Direct role for Myc in transcription initiation mediated by interactions with TFII-I. Nature **365**:359-361.

105. **Sakamuro, D. and G. C. Prendergast**. 1999. New Myc-interacting proteins: a second Myc network emerges. Oncogene **18**:2942-2954.

106. **Sambrook, J., E. F. Fritsch, and T. Maniatis**. 1989. Expression of cloned genes in cultured mammalian cells, p. 16.60-16.62. *In* N. Ford, C. Nolan, and M. Ferguson (ed.), Molecular Cloning: A laboratory Manual. Cold Spring Harbor Laboratory Press, New York.

107. **Sambrook, J., E. F. Fritsch, and T. Maniatis**. 1989. Expression of cloned genes in cultured mammalian cells, p. 16.41-16.44. *In* N. Ford, C. Nolan, and M. Ferguson (ed.), Molecular Cloning: A laboratory Manual. Cold Spring Harbor Laboratory Press, New York.

108. **Sambrook, J., E. F. Fritsch, and T. Maniatis**. 1989. Expression of cloned genes in cultured mammalian cells, p. 16.33-16.36. *In* N. Ford, C. Nolan, and M. Ferguson (ed.), Molecular Cloning: A laborator Manual. Cold Spring Harbor Laboratory Press, New York.

109. **Sambrook, J., E. F. Fritsch, and T. Maniatis**. 1989. Detection and Analysis of Proteins Expressed from cloned genes, p. 18.30-18.46. *In* C. Nolan, M. Ferguson, and N. Ford (ed.), Molecular Cloning:A laboratory Manual. Cold Spring Harbor Laboratory Press, New York.

110. **Sambrook, J., E. F. Fritsch, and T. Maniatis**. 1989. Detection and analysis of proteins expressed from cloned genes, p. 18.47-18.54. *In* N. Ford, M. Ferguson, and C. Nolan (ed.), Molecular Cloning: A laboratory Manual. Cold Spring Harbor Laboratory Press, New York.

111. **Seed, B. and J. Y. Sheen**. 1988. A simple phase extraction assay for chloramphenicol acetyltransferase activity. Gene **67**:271-277.

112. **Seto, E., Y. Shi, and T. Shenk**. 1991. YY1 is an initiator sequence-binding protein that directs and activates transcription in vitro. Nature **354**:241-245.

113. **Shrivastava, A., S. Saleque, G. V. Kalpana, S. Artandi, S. P. Goff, and K. Calame**. 1993. Inhibition of transcriptional regulator Yin-Yang-1 by association with c-Myc. Science **262**:1889-1892.

114. **Shrivastava, A. and K. Calame**. 1994. An analysis of genes regulated by the multi-functional transcriptional regulator Yin Yang-1. Nucleic Acids Res. **22**:5151-5155.

115. **Shrivastava, A., J. Yu, S. Artandi, and K. Calame**. 1996. YY1 and c-Myc associate in vivo in a manner that depends on c-Myc levels. Proc.Natl.Acad.Sci.U.S.A **93**:10638-10641.

116. **Sommer, A., H. Burkhardt, S. M. Keyse, and B. Luscher**. 2000. Synergistic activation of the mkp-1 gene by protein kinase A signaling and USF, but not c-Myc. FEBS Lett. **474**:146-150.

117. **Sun, Y. and E. A. Clark**. 1999. Expression of the c-myc proto-oncogene is essential for HIV-1 infection in activated T cells. J.Exp.Med. **189**:1391-1398.

118. **Sundseth, R. and U. Hansen**. 1992. Activation of RNA polymerase II transcription by the specific DNA-binding protein LSF. Increased rate of binding of the basal promoter factor TFIIB. J.Biol.Chem. **267**:7845-7855.

119. **Tang, H., K. L. Kuhen, and F. Wong-Staal**. 1999. Lentivirus replication and regulation. Annu.Rev.Genet. **33**:133-170.

120. **Tasara, T., M. O. Hottiger, and U. Hubscher**. 2001. Functional genomics in HIV-1 virus replication: protein-protein interactions as a basis for recruiting the host cell machinery for viral propagation. Biol.Chem. **382**:993-999.

121. **Thomas, M. J. and E. Seto**. 1999. Unlocking the mechanisms of transcription factor YY1: are chromatin modifying enzymes the key? Gene **236**:197-208.

122. **Trono, D.** 1995. HIV accessory proteins: leading roles for the supporting cast. Cell **82**:189-192.

123. **Turner, B. G. and M. F. Summers**. 1999. Structural biology of HIV. J.Mol.Biol. **285**:1-32.

124. **Usheva, A. and T. Shenk**. 1994. TATA-binding protein-independent initiation: YY1, TFIIB, and RNA polymerase II direct basal transcription on supercoiled template DNA. Cell **76**:1115-1121.

125. **Valvatne, H., A. M. Szilvay, and D. E. Helland**. 1996. A monoclonal antibody defines a novel HIV type 1 Tat domain involved in trans-cellular trans-activation. AIDS Res.Hum.Retroviruses **12**:611-619.

126. **Watson, K. and R. J. Edwards**. 1999. HIV-1-trans-activating (Tat) protein: both a target and a tool in therapeutic approaches. Biochem.Pharmacol. **58**:1521-1528.

127. **Weiss, R.** 2000. Getting to Know HIV. Tropical Medicine and International Health **5**:A10-A15.

128. **Yang, B. S., T. J. Geddes, R. J. Pogulis, B. de Crombrugghe, and S. O. Freytag**. 1991. Transcriptional suppression of cellular gene expression by c-Myc. Mol.Cell Biol. **11**:2291-2295.

129. **Yankulov, K. and D. Bentley**. 1998. Transcriptional control: Tat cofactors and transcriptional elongation. Curr.Biol. **8**:R447-R449.

130. **Yoon, J. B., G. Li, and R. G. Roeder**. 1994. Characterization of a family of related cellular transcription factors which can modulate human immunodeficiency virus type 1 transcription in vitro. Mol.Cell Biol. **14**:1776-1785.

131. **Zeichner S.L., Kim J.Y.H., and Alwine J.C.** 1991. Linker-Scanning Mutational Analysis of the Transcriptional Activity of the Human Immunodeficiency Virus Type 1 Long Terminal Repeat. Journal of Virology **65**:2436-2444.

132. **Zhao, J. H., T. Inoue, W. Shoji, Y. Nemoto, and M. Obinata**. 1998. Direct association of YY-1 with c-Myc and the E-box binding protein in regulation of glycophorin gene expression. Oncogene **17**:1009-1017.