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Regulation of HIV-1 postintegration latency by NF-kappa-B

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Regulation of HIV-1 Postintegration Latency by NF-kappa-B

by

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DISSERTATION

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This work is dedicated to my parents, whose love of knowledge, respect for academic pursuit, and guidance in all manners of life made these studies possible.

Preface

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The text of Chapter 2 is a reprint of a research article published in *the Journal of Biological Chemistry*. This article was published by Samuel A. Williams, Lin Feng Chen, David Fenard, Dwayne Bisgrove, Eric Verdin, and Warner C. Greene. The text of chapter 3 is published as a research article at the *EMBO Journal*. This article was authored by Samuel A. Williams, Lin Feng Chen, Hakju Kwon, Carmen Martin Ruiz Jarabo, Eric Verdin, and Warner C. Greene. Chapter 4 contains a manuscript in preparation for possible publicaton in a scientific journal. The text of Appendix I is a reprint of a research focus article published in *Trends in Microbiology*. The article was authored by Samuel A. Williams and Warner C. Greene.

Regulation of HIV-1 Postintegration Latency by NF-κB

The past two decades of HIV research have transformed the diagnosis of HIV-1 infection from a death sentence to a clinically manageable life-long disease. Despite intensified efforts to identify more diverse and efficient inhibitors of viral replication, resistant reservoirs of HIV-1 continue to thwart viral eradication, and force patients to remain on costly, and often toxic regimens of drugs. Among the reservoirs contributing to long-term persistence of HIV-1 in the face of effective therapy is a pool of latently infected memory CD4 T-cells. These cells harboring integrated but transcriptionally inactive proviruses can persist for decades while retaining the potential to activate viral production and reseed systemic infection when antiviral therapy is discontinued. We have sought to understand in molecular terms the bases for HIV latency with an eye to identifying novel strategies to purge the latent reservoir. While undoubtedly a difficult problem, success in this effort could realize the long-sought goal of a cure for HIV-1 infected patients.

$CD4^+$ T-cells harboring latent HIV-1 are very rare and lack any distinguishing markers. As a consequence, these cells are exceedingly difficult to isolate from infected patient blood. To systematically explore the molecular events underlying HIV-1 latency, we made use of the Jurkat $CD4^+$ T cell based J-Lat model of HIV-1 latency. The J-Lat model is comprised of several clonally selected cell lines that each harbor a single integrated HIV-1 provirus that fails to express viral genes. Viral gene expression can be induced within the latently infected J-Lat clones by a variety of cell-activating stimuli.

In our initial study, we identified the phorbol ester prostratin as an inducer of latent HIV-1 gene expression in the J-Lat model of latency. We demonstrated a central role for the NF-κB family of transcription factors as mediators of prostratin antagonism of HIV, and for the first demonstrated recruitment of NF-κB RelA to the HIV-1 LTR *in vivo*. These studies prompted us to investigate in more detail the processes regulated by NF-κB that modulate expression of latent HIV-1.

Further examination of the J-Lat system revealed that HIV-1 latency is reinforced by the binding of a NF-κB1 p50-HDAC repressor complex to the latent HIV-1 LTR. This complex induces localized histone deacetylation, which restricts RNA polymerase II access to the HIV-1 LTR, thereby preventing initiation of HIV mRNA synthesis. siRNA knock down studies demonstrated a key role for p50 in the exclusion of RNA polymerase II from the latent HIV-1 LTR, and chemical inhibition of HDAC activity similarly promoted polymerase binding.

In a third and concluding study, we examined the kinetics of NF-κB mediated induction of latent HIV-1 gene expression. These studies describe a dynamic process of transcriptional regulation induced by NF-κB, and suggest that sustained NF-κB activity is required for efficient activation of latent HIV-1 gene expression. Within the context of latent HIV-1 infection, NF-κB appears to be a strong inducer of HIV-1 transcriptional initiation, but fails to promote efficient elongation. While NF-κB induction alone appears to be insufficient to induce strong HIV-1 gene expression, supply of HIV-1 Tat in *trans* strongly induces transcription.

In summary, our studies have uncovered a new range of biology at the interface of NF-κB and the regulation of HIV-1 gene expression. The findings of our studies imply

that NF-κB-inducing agents administered in conjunction with HDAC inhibitors or HIV-1 tat could be strong activators of latency in infected patients. These studies are likely to be the basis of new inroads into the understanding of events regulating transcriptional repression and activation of the latent HIV-1 LTR.

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Chapter 1

Introduction

I. OVERVIEW

The advent of highly active antiretroviral therapy offered early hope that a cure to HIV-infected patients might be in the offing. Antiviral drug cocktails have achieved remarkable success in suppressing viral replication, often to undetectable levels. However, these targeted therapies have been unable to eradicate virus altogether from infected patients. As a consequence, existing therapies require lifelong administration; any interruptions result in a rapid rebound of virus and subsequent decline in CD4+ T-cell counts. A major source of this rebounding virus is from a pool of latently infected CD4+ T-cells, which harbor dormant virus in a stable form that can endure for decades. Due to its non-replicative nature, latently infected T-cells are not affected by antiviral drugs. Despite their dormancy, latently infected T-cells retain the potential to produce infectious when activated by specific antigen or various cytokines.

HIV replication is closely wed to T-cell activation through a wide range of inducible transcription factors including AP-1, NFAT, and NF-κB. Antigen- or soluble cytokine-mediated activation of CD4+ T-cells drives the induction of several intracellular signaling pathways, activating the NF-κB family of transcription factors. Activated NF-κB complexes bind to κB-enhancer DNA elements and regulate expression of cellular genes. HIV-1 expropriates these cellular transcription factor complexes through dual κB-enhancer elements present in proviral regulatory DNA to direct viral gene expression. The precise events driven by NF-κB to enhance HIV gene expression are not entirely understood. Several members of the NF-κB/Rel family of transcription factors are capable of binding regulatory elements contained in HIV's enhancer *in vitro*. Which of these factors bind to the HIV enhancer *in vivo*, when, and how they exert control over

HIV transcription remain open questions. These studies were designed to elucidate the role of NF-κB in the context of post-integration HIV latency with primary focus on the interplay of NF-κB transcription factors with the regulatory elements controlling HIV expression. A secondary focus was an exploration of the basic transcriptional machinery and chromatin status associated with the latent and transcriptionally active integrated HIV promoters. The results of this work have extended our understanding of HIV transcriptional biology in both the latent and activated states, and point to new strategies to attack the latent reservoir.

II HIV-1 Life Cycle

HIV, the etiological agent responsible for acquired immunodeficiency syndrome (AIDS) [1], is a member of the lentivirus group of the Retrovirus family [2]. It is genetically similar to viruses observed in simian populations, and is thought to have entered the human population through a zoonotic transmissions, perhaps in the 1930s [3]. Though many individual transmission events may have occurred, two transmission events are clearly discernable, as evidenced by the similar but genetically distinct HIV-1 and HIV-2. HIV-1, responsible for the vast majority of the HIV pandemic, is most similar to a simian immunodeficiency virus (SIV) observed in chimpanzees [4]. By contrast, HIV-2 appears to be more similar to SIV endemic to sooty mangabeys [5], and for unclear reasons appears to have largely remained geographically constrained within populations in Western Africa and India [6].

The molecular event surrounding the lifecycle of HIV-1 have been elucidated in great detail over the last 25 years (see [7] for review). As with other retroviruses, free

HIV-1 virions are enveloped with a lipid bilayer membrane derived from the producer cell and contain an RNA genome surrounded by virally-encoded nucleocapsid and matrix proteins [8]. Virally encoded gp41/gp120 envelope proteins protruding from the viral envelope bind to cellular CD4 receptors present on the surface of target cells, initiating the cycle of infection [9, 10]. Following CD4 binding, conformational changes induced in gp120 expose the coreceptor binding site which recognizes various cellular chemokine receptors, commonly CCR5 or CXCR4 [11-14]. Upon binding of the chemokine coreceptor, a further conformational change of gp120 is induced, resulting in the insertion of the amino-terminal fusion peptide gp41 into the target cell, and fusion of the viral and cellular membranes [15]. Following fusion, the contents of the virion are "injected" into the cytoplasm through the fusion pore, where a still-poorly understood process of viral capsid disassembly occurs. Virally-encoded reverse transcriptase transported to the target cell within the incoming virion reverse-transcribes the RNA genome to a DNA species which is transported into the nucleus in a process facilitated in part by the virally encoded Vpr, Matrix, and Integrase proteins [16]. Following nuclear import, the virion-associated virally encoded integrase facilitates insertion of the viral DNA genome into host genomic DNA. This process appears to be largely random with respect to locus of integration [17], though sites of active transcription appear to be favored relative to areas of reduced transcriptional activity [18-20]. The site of integration plays a key role in the basal transcriptional activity of the provirus, and can predispose an integrated provirus to a latent state. Once integrated, the viral genome exists in a stable form that can persist indefinitely, enduring for the life of the host cell.

The integrated HIV-1 genome contains at its 5' and 3' ends repeated genetic elements aptly termed long terminal repeats, a consequence of strand transfer during the reverse transcription process [8]. The 5' LTR encodes a series of regulatory elements that directs transcription of viral genes: *gag (CA, MA, p6, p7)*, *pol (PRO, RT, INT)*, *env (gp190, gp41)*, *tat*, *rev*, *nef*, *vpr*, *vpu*, and *vif* [21]. A single viral mRNA is produced, which is differentially spliced to encode each of the various viral proteins, with the exception of *pol*. This gene product is produced as a gag-pol fusion by a process involving stochastic translational frameshifting [22]. In the early phase of HIV-1 gene synthesis, the *tat*, *rev*, and *nef* gene products predominate as full-length transcripts are rapidly spliced [23, 24]. Tat binds to an RNA stem loop structure termed TAR present in the 5' end of nascent HIV mRNA transcripts and promotes increased transcriptional activity (discussed in detail in section IV) [25]. Nef directs down-regulation of surface CD4 and MHC-I antigen presenting molecules, promotes enhanced sensitivity to cellular activation signals, and enhances infectivity of produced virions [26-30]. Rev binds to a rev-responsive element (RRE) contained in full-length HIV mRNA transcripts, and promotes Exportin-1 /Ran GTP-dependent RNA export [31]. This prevents nuclear splicing of genomic HIV-1 RNA, and preserves the long transcript for synthesis of *gag*, *pol*, *env*, and other accessory gene products. Viral Env is translated as a 160 kD transmembrane protein and processed by the cellular protease furin to form the gp120 and gp41 Env proteins, which are exported to the cellular or lysosomal compartments for eventual virion incorporation [8]. The amino-terminal region of Gag and Gag-Pol molecules are myristolated, a modification that drives these molecules to cellular membranes [32]. Gag and Gag-Pol then cluster in a HP68/ATP-dependent, ordered

process into previrion capsid particles [33]. Two zinc-finger motifs present in the p7 nucleocapsid subunit of Gag recruit HIV genomic RNA to the forming virion [34]. Newly formed virions bud from the surface of infected cells with the aid of TSG101, a cellular vacuolar ATPase, which is recruited by a late domain in the p6 subunit of Gag [35]. Viral budding is further promoted through downregulation of the cellular CD4 receptor by Vpu, Nef , and Env [26, 36]. Vif enhances outgoing virion infectivity by directing Cul5-dependent proteosomal degradation of APOBEC3G [37-42]. In the absence of Vif, this cellular cytidine deaminase is incorporated into HIV-1 virions where it induces lethal hypermutation of incoming viral genomes [43-45].

The net effect of this highly orchestrated series of events is the amplification of infectious viral particles through each round of the viral life cycle, propagating infection throughout the host. Studies of viral transmission suggest that dendritic cells (DCs) are among the first targets of HIV within newly infected hosts [46-48]. Immature DCs express both CD4 and CCR5, and support the complete HIV life cycle [49]. These cells participate in immune surveillance and monitor mucosal surfaces for potential pathogens (see [50] for review). Upon activation DCs migrate to local lymph nodes where they present antigens to CD4+ and CD8+ lymphocytes for potential immune response. HIV is thought to exploit this aspect of DC biology, using these immune cells as a "Trojan horse" to transmit virus to activated CD4+ cells [51, 52]. DCs infected with HIV can produce infectious virus for months, or alternatively, virus can be retained on DC-SIGN or other surface glycoproteins in a highly stable form [53-55]. In either case, these cells represent a stable and highly effective source of HIV production.

Though they are of primary importance in the context of HIV-associated disease, CD4+ T-cells can be thought to be secondary targets of HIV in the context of viral transmission [47, 48]. The majority of CD4+ T-cells circulating in the blood and lymph are in a resting state that is not permissive for HIV infection. Viruses that fuse to resting CD4+ T-cells fail to complete reverse transcription [56, 57] or integrate [58], because of interference by low molecular mass APOBEC3G present in the target cell [59]. The mechanism employed by APOBEC3G to impair replication in resting cells is not yet clear, but is likely either a consequence of direct interference with RT's processing of HIV RNA, or by hypermutation of the incoming viral genomic RNA, or both. In contrast, CD4+ T-cells activated by specific antigen, CD3-crosslinking, or various cytokines present highly permissive targets to infection with HIV. The block posed by APOBEC3G is overcome by its recruitment into a high molecular mass RNA-protein complex. Establishment of infection within the T-cell compartment leads to increased levels of T-cell activation [60], as the immune system attempts to rebuff the systemic dissemination of HIV. Macrophages, CD4+ natural killer cells, as well occasional CD8+ or B-cells eventually become infected as HIV begins its exponential advance (reviewed in [61]).

Titers of virus often reach to levels of 10^7 copies per milliliter of plasma in the first weeks of infection, and CD4 T-cell counts decline rapidly [62-64]. This initial peak in viremia usually drops sharply as the immune response partially contains the virus to a steady state level of viremia 100-1000x less than the viral peak [65]. This stable level of viremia, termed the viral "set-point", is a strong predictor of the rate of disease progression [66]. Concurrent with the resolution of acute HIV viremia, rebound of CD4

T-cell counts is typically observed. For a period of time, often lasting years, a period termed "clinical latency" ensues, characterized by the absence of overt symptoms of HIV infection. CD4 T-cell counts progressively decline with concomitant rise in viral titers, leading eventually to breakthrough in viremia and rapid depletion of CD4+ T-cells. Analyses of lymph nodes of infected patients indicate that the majority of dying CD4+ T-cells are not infected with HIV [67]. Rather, "bystander killing" appears to play a dominant role in progressive CD4+ T-cell depletion. The end result of the depletion of CD4+ T-cells is the collapse of the immune system, manifesting as various opportunistic infections and cancers that ultimately lead to the demise of the patient.

Early efforts to interfere with viral replication focused on development of inhibitors of HIV reverse transcription. AZT, a nucleoside reverse transcriptase inhibitor, demonstrated an ability to inhibit viral replication in tissue culture settings [68]. Early patients treated with AZT monotherapy experienced rapid reductions in viral load [69]. However, this reduction proved to be exceedingly transient, and AZT-resistant HIV usually emerged [70, 71]. The error-prone process of reverse transcription proved to be a remarkably efficient mechanism for generating a "swarm" diverse viruses [72, 73]. Indeed, viruses with mutations conferring resistance to various drugs likely pre-exist within the swarm and emerge when drug administration promotes its selective growth. Mathematical models predict that in the course of a single day every possible point mutation possible is generated in an infected patient, and that every double mutation is generated in the span of three months [74]. Subsequent monotherapy efforts targeting HIV protease or reverse transcriptase with nonnucleoside inhibitors were similarly undermined by rapid emergence of resistant virus [75, 76]. Highly active antiretroviral

therapy (HAART), a combination of HIV protease and reverse transcriptase inhibitors, proved to be the first effective means of durably suppressing HIV viral load and increasing CD4+ T-cell counts in infected patients [77, 78].

III HIV-1 LATENCY

Early studies of viral decay kinetics following initiation of HAART in infected patients demonstrated that viral titers decline in a biphasic pattern. An initial period of exponential decline lasts ~2 weeks, likely reflecting rapid turnover of free virions and productively infected cells. This initial period is followed by a secondary linear decay phase with indeterminate duration, likely reflecting the turnover of longer-living infected cells including macrophages and dendritic cells [79-82]. The second phase of viral decay often reduces viral titers below the limit of detection of standard assays[1], prompting many to suggest that this therapy would ultimately eradicate the virus in infected patients [85, 86]. Mathematical models based on these early studies suggested that viral eradication might be achievable with as little as 2-3 years of continuous administration of HAART [79].

Long-term studies revealed that cessation of HAART, with exceedingly rare exception [87], leads to rebound of viral of plasma viral levels within a few weeks of drug withdrawal despite durable suppression of virus below detectable levels during prior therapy [88-90]. This rebound suggested either an incomplete inhibition of viral replication by the therapeutic regimen, or the existence of viral reservoirs: cellular or

[1] In 1996, the time of these studies, the Roche Amplicor HIV-1 Monitor RT-PCR test was the standard, with a detection limit of 400 copies/ml [83]. In 1998, Roche released an "UltraSensitive" RT-PCR HIV-1 quantitation kit with a detection limit of 50 copies/ml [16]. More recent developments have pushed this limit to less than a single viral copy per milliliter [84]. Analyses of patients with "undetectable viral loaf" with more sensitive assays has generally identified virus present at levels less than the former limit.

anatomical compartments that harbor replication-competent virus in an environment resistant to antiretroviral therapy. Several reservoirs of long-lived HIV had been predicted prior to these studies, including virus bound to follicular dendritic cells [54], infected macrophages in the CNS [91] and latently infected[2] resting CD4+ T-cells [92]. The CNS provides an example of an anatomically privileged locus of infection, where supra-therapeutic levels of antiretroviral drugs are observed due to active drug export across the blood-brain barrier [93, 94]. Latently infected CD4+ T-cells, however, reside in the blood and lymphatics where antiretroviral drugs exist at strongly inhibiting concentrations. This reservoir persists not because of an insufficiency of inhibitory drugs, but rather because the virus contained within these infected cells exists in a dormant state where the viral protein targets of antiretroviral therapy are not synthesized. Thus, while other reservoirs of HIV-1 can be confronted with intensification of therapy or improved local delivery of antiretroviral drugs, the pool of CD4+ cells harboring latent virus requires a more radical approach.

Rebounding virus following HAART interruption shares genetic similarity with virus contained in the pool of latent HIV-1 [95]. In contrast, no such similarity has been noted among other prominent reservoirs, including CNS macrophages, or follicular dendritic-cell associated virions, though one study found no similarity to any known latent pool [96]. These observations bolster the claim that CD4+ lymphocytes harboring latent proviruses are key contributors to the reemergence of viremia following HAART cessation. HIV-1 detection assays with improved sensitivity have identified continued

[2] Latent viral infection is a distinct concept from clinical latency, described in section II. The former refers to a protracted delay separating establishment of infection of a cell and the subsequent progression to synthesis of viral gene products, wile the latter refers to the symptom-free period of infection commonly observed after acute infection and prior to the onset of AIDS.

low-level viremia in HAART-suppressed patients with previously undetectable titers [97], suggesting that incomplete suppression of viral replication may also contribute to the rapid rebound of viral titers. However, the eventual predominance of wild-type non-drug-resistant virus with genetic similarity to virus found early in infection, highlights the central contribution of the durable pool of latently infected CD+ T-cells to this rebound.

Among the drug-resistant reservoirs of HIV-1, the pool of latently infected CD4+ memory T-cells appear to have the longest lifespan, with a minimal half-life of 44 months [98-100]. Models predict that clearance of this pool will require 50-60 years of continued viral suppression, a period roughly approximating that of the expected life span of the infected patient. The stability of this pool is owed in large part to the nature of the infected cell, the memory CD4+ T-cell. Memory T-cells are produced in the wake of the typical T-cell immune response; following antigenic activation of a naive T-cell, a cascade of proliferative cell division and differentiation is initiated producing two distinct classes of immune cells. The more abundant effector T-cells actively participate in the antigen-directed immune response, whereas memory T-cells mediate long-term term immunity (reviewed in [101]). Memory CD4+ T-cells have long life spans in healthy patients, a feature that contributes to the "life-long" immunity to many childhood diseases. The pool of CD4+ memory T-cells is maintained in a process of homeostatic renewal in which rare cell division occurs [102]. This issue may be key to the targeted elimination of latent HIV-1.

Siliciano and Chun first identified CD4+ T-cells harboring latent HIV-1 in patients 1995 [92]. Resting CD4+ T-cells were isolated from blood of infected patients, incubated for several days in the presence of antiretroviral drugs to eliminate any residual

active infection. These cells were then stimulated with PHA and IL-2 to promote T-cell activation and outgrowth of latent virus. Heterologous activated CD4+ T-cells were added as target cells to amplify infection, and outgrowth of replication-competent latent virus was universally observed after 1-3 weeks of culture. Serial dilution studies found that the average frequency of latent infection is approximately 1-4 cells infected with replication competent HIV-1 per million CD4+ T-cells isolated, or about 10^6 latently infected cells per patient [103]. This stands in contrast to the observed frequency of 100-600 cells containing integrated HIV-1 proviral DNA per million CD4+ T-cells [103]. Together, these observations indicate that the majority of integrated virus is either defective due to inactivating mutations within the viral genome or the site of integration within the target cell, or that alternative stimulation is required for viral activation within these cells. Subsequent studies demonstrated that the pool of latent HIV-1 infection is established early in the course of disease, likely during acute infection [92].

The processes underlying the generation of replication competent latent HIV-1 infection remain unclear. Multiple parallel pathways are likely to be involved. In view of the potent post-entry block of resting T-cells to HIV-1 infection caused by LMM APOBEC3G, it is likely that activated T-cells are the ultimate source of latent infection. Because activated T-cells are capable of supporting the full HIV-1 life cycle, it is unclear why some activated cells should become latently infected while the majority become actively infected. Infection of incompletely activated T-cells, supporting early but not late events of the viral life cycle may account for some viral latency. Alternatively, fully activated T-cells may be infected during the resolution of antigenic activation, providing a short window permitting reverse transcription and integration but precluding activation-

dependant viral gene expression. Observations of latent HIV-1 present in both naive and memory T-cells provide evidence for both routes of latency generation [103]. Other models include the possibility of infection of $CD4^+CD8^+$ T-cells during activation associated with thymic generation [104], however, infection of $CD8^+$ cells as predicted by this model, though reported [105], does not appear to be common. Whichever the route of generation, each scenario is predicted to produce integrated HIV-1 provirus requiring cellular activation for rescue of the viral lifecycle.

HIV-1 viral latency is defined by a failure of viral gene expression in an infected cell that is recoverable by cellular activation. Forms of HIV-1 latency fall into two broad classes based on the progression of the infected cell through the lifecycle relative to proviral integration. In preintegration HIV-1 latency, the viral provirus exists as an extra-chromosomal episome, failing to integrate into the host genome due to reverse transcription, nuclear import, or integrase defects. Cellular activation permits progression of the provirus through the pause in lifecycle, promoting proviral integration and subsequent gene expression [57, 103]. This form of provirus is relatively labile, and is subject to degradation with a half-life of 2-3 days [106]. In postintegration HIV-1 latency, the provirus proceeds through reverse transcription, nuclear import and integration into the host genomic DNA, however, the life cycle then halts prior to *de novo* viral synthesis. After proviral integration, the proviral genome is maintained by cellular DNA repair machinery, and thus can persist for the lifetime of the cell.

HIV-1 does not appear to explicitly direct the generation of a latent state, as observed in other viruses such as Kaposi's Sarcoma or HSV. Mechanisms accounting for the failure of gene expression observed in postintegration viral latency include proviral

mutations, transcription factor insufficiency, and integration into transcriptionally unsupportive regions of the host genome. Point mutations in proviral DNA can impair the viral gene expression by introducing premature stop codons or missense mutations that disrupt the normal function of viral genes. Mutations within *tat* or *rev*, or their respective TAR or RRE RNA targets impair the ability of these viral proteins to regulate gene expression [107-110]. While in most cases such mutations would produce replication incompetent viruses, isolated incidences of activation-inducible HIV-1 containing mutations in *tat* [111] or TAR have been identified in cell culture [112]. These integrated proviruses contain strongly debilitating mutations, however, the cells are capable of producing infectious virus upon activation. Thus, these mutated viruses are one source of viral latency. Given the rate of mutation during reverse transcription, it is probable that point-mutant reversions resulting in wild-type infectious virus could be generated from such a latent source.

Memory CD4+ T-cells circulate in a resting state characterized by reduced metabolic activity, and arrest in the G_0 stage of the cell cycle (see [113] for review). This state of quiescence is well suited to the task of passive immune surveillance, and ensures that the inactive immune system is efficient in energy consumption. In general, cellular machinery promoting transcription is reduced relative to more metabolically active cells, and is consequently limiting for extraneous gene expression. Reduced levels of CDK9 and cyclinT1, cellular cofactors of HIV gene expression have been implicated as key determinants of viral latency in resting CD4+ T-cells [114, 115]. Resting T-cells are also distinguished from their activated cellular counterparts by reduced nuclear levels of

various T-cell activation-inducible transcription factors known to promote HIV gene expression including AP-1, NFAT, and NF-κB (discussed in further detail in section IV).

In some cases, the site of HIV-1 proviral integration appears to be an additional determinant of postintegration latency. Clear evidence supports the notion that the level of basal HIV-1 gene expression is directly regulated by *cis*-acting DNA elements surrounding the provirus [116]. Integration into regions of silenced heterochromatin near telomeres, centrosomes, and alphoid-repeats has a strong silencing effect on basal HIV-1 transcription, and promotes the likelihood of latency [117]. Paradoxically, insertion into highly expressed host genes also appears to promote viral latency, likely through a promoter interference [118]. Increasing evidence points to an important role for the chromatin environment surrounding the integrated provirus in the regulation of HIV gene expression, though little is known of the processes directing this. Sodium butyrate and valproic acid, inhibitors of chromatin modifying histone deacetylases (HDACs), induce activation of latent HIV-1 in both cell culture models and blood isolated from HAART suppressed HIV-infected patients [119, 120]. Direct methylation of HIV genomic DNA has also been proposed to suppress expression of latent provirus [121], however, this modification has not been observed in models of latency [122].

Recognizing that latent HIV-1 requires cellular activation to induce viral gene expression, several groups have investigated the effects of general T-cell activators in infected HAART-suppressed patients. This strategy aims to induce viral gene expression and expose latently infected cells to antiretroviral therapies. Efforts with OKT3, a CD3-stimulating antibody that mimics antigenic activation, and IL-2, a T-cell activating cytokine, proved to be ineffective in reducing the size of the latent pool, due largely to

the negative side effects of their generalized immune activating effects [123]. Milder approaches employing IL-2 alone [124-126] or with other cytokines [127] have proven less toxic, but have not demonstrated any substantial reduction in the size of the latent pool of HIV-1. The design of effective approaches to the daunting problem of HIV-1 latency is hampered by a lack of understanding of the molecular basis for such latency. A direct aim of this work was to characterize the molecular events induced by T-cell activating agents that drive latent HIV-1 gene expression (Chapter 2).

IV Transcriptional Regulation of HIV-1

HIV-1 gene expression is regulated principally at the level of transcription, and is directed by virally-encoded promoter and enhancer elements contained within the 5' LTR [128]. The core promoter, comprised of three binding sites for the constitutive cellular transcription factor SP1, a TATA box, and an initiator sequence, binds the cellular preinitiation complex comprised of TBP, TFIIB, TFIID, and the RNA polymerase II holoenzyme [129]. *In vitro* studies indicate that the core promoter element is sufficient for HIV-1 transcriptional initiation [130], however, transcripts initiated in this context are routinely terminated as short ineffective transcripts.

Early studies of HIV transcriptional regulation identified the early viral protein tat as a strong transactivator of HIV gene expression [131]. Tat binds to the TAR stem loop structure present at the 5' end of all initiated RNA transcripts and promotes transcriptional elongation [25, 132]. Whether Tat affects the rate of transcriptional initiation is still a point of contention. The elongating function of Tat, however, is commonly accepted. Tat recruits the cellular positive transcription complex p-TEFb,

comprised of cylinT1 and CDK9, which phosphorylates a repeated region present in the carboxy-terminal domain (CTD) of RNA Pol II. This modification stabilizes the transcriptional processivity of RNA Pol II [133-136]. Mutations of Tat that ablate p-TEFb binding strongly correlate with a loss of HIV transactivation [137]. Additionally, mutation or deletion of the CTD of RNA Pol II strongly reduces Tat transactivation [138]. Tat transactivation of HIV-1 transcriptional elongation is strongly inhibited by the nucleoside analog CDK-kinase inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), supporting a role for CDKs as elongation cofactors in HIV transcription [139]. p-TEFb further promotes transcriptional elongation by phosphorylating SPT5 in the cellular N-TEF transcriptional repressor complex comprised of DSIF and NELF, ablating the repressive nature of these negative transcriptional regulators [140].

Recent studies by Michael Green indicate that Tat can transactivate HIV in the absence of TAR [141]. Though it is not clear how Tat specifically targets the HIV promoter in this model, it has been suggested that Tat-associated p-TEFb drives increased recruitment of TBP to the HIV promoter. This, in turn directs increased transcriptional preinitiation complex formation, and enhances transcriptional initiation. Whether HIV-1 latency *in vivo* is more commonly a consequence of failures in transcriptional initiation or elongation will be of central importance to eradication efforts. One study that attempted to analyze the transcriptional activity of the CD4+ T-cell pool harboring latent HIV-1 in infected patients identified robust levels of short, but not long HIV mRNA transcripts, suggesting that the absence of key elongating cofactors might underlie HIV latency [142]. However, this study did not discriminate between latently infected cells, and cells

infected with far more common defective proviruses, thus confounding clear interpretation.

Mammalian DNA is compacted and organized into chromatin through interactions with structural proteins, termed histones. Octomeric assemblies of various histone proteins package 180 nucleotide stretches of DNA into repetitive coils termed nucleosomes [143, 144]. Upon integration, the HIV provirus is rapidly assembled into precisely positioned nucleosomes, one of which directly overlaps the transcriptional initiation site as well as the coding sequence for TAR, nuc-1 [145]. In the absence of stimulatory signals, the chromatinized LTR remains transcriptionally repressed [146]. HIV T-cell activation promotes a specific repositioning or rearrangement of the nucleosomes into which the HIV promoter is organized, changes which are thought to promote increased availability to transcriptional machinery [145]. Nucleosomal remodeling is a consequence of post-translational modification of histone proteins, including acetylation, phosphorylation, methylation, and ubiquitylation, among others, driven by the localized recruitment of various histone acetyltransferses (HATs), histone deacteylases (HDACs), kinases, phosphatases, etc. (reviewed in [147]). Patterns of histone modifications appear to be associated with transcriptional activation and repression, with hyperacetylation favoring the former, and methylation promoting either depending on specific the site of modification. Histone hyperacetylation driven by HDAC inhibitors induces reorganization of nucleosomes in the HIV LTR, and promotes transcriptional activation of HIV in many model systems, suggesting a key role for histone acetylation in the regulation of HIV transcription [148, 149]. Purified acetyltransferases markedly stimulate transcriptional activity of *in vitro* chromatin

assemblies of the HIV promoter, supporting a positive role for histone acetylation in HIV transcriptional activation [150].

Several HDACs and HATs have been implicated in the modulation of HIV-1 transcription. Tat mediated recruitment of p300/CBP and PCAF to the HIV-1 LTR, where they promote hyperacetylation of local histones [146, 151, 152]. Tat itself is a target for acetylation by these enzymes, and dynamic modulation of Tat acetylation appears to be a key for its transactivation of HIV-1 [153]. HDAC1 has been implicated as a histone deacetylases that modulates the HIV promoter and promotes chromatin condensation and repression of HIV transcription [154]. LSF1, YY1, and thyroid hormone receptor recruit HDAC1 to the HIV-1 promoter through cognate binding sites present in the LTR [154, 155]. The overall contribution of each of these factors to HDAC1 recruitment and transcriptional repression, however, has not been thoroughly examined.

The ATP-dependant chromatin remodeling SWI/SNF complex has been proposed to be a key factor in the elongation of RNA Pol II, and is inducibly recruited to the HIV promoter following cellular stimulation with T-cell activators [156]. Whether p-TEFb synergizes with SWI/SNF to regulate transcriptional processivity from the HIV-1 promoter has not been examined. *In vitro* studies of nucleosome-assembled transcription targets indicate that nucleosomes strongly inhibit transcriptional initiation, however, once initiated, RNA Pol II is able to extend through nucleosomes with little difficulty [157-159].

The reduced rate of transcriptional elongation and synthesis of full-length HIV mRNA transcripts in the absence of Tat presents a basic problem for the newly infected

cell: how are initial elongated viral transcripts synthesized such that Tat itself can be produced? Transcriptionally active cellular promoters proximal to the locus of proviral integration may transactivate the HIV promoter in *cis*, providing low levels of activating signals that are sufficient for reduced rates of transcriptional elongation [160]. Alternatively, the block to elongation in the absence of Tat may not be absolute, with initiated transcripts occasionally elongating to full length, thus eventually permitting Tat synthesis. Finally, cellular cofactors that functionally mimic Tat may bind to the HIV promoter and promote analogous elongating function. Indeed, it appears that each of these processes contribute to the initiation of Tat synthesis, however, the latter option appears to be the most important for the resolution of HIV-1 latency.

The 5' LTR contains binding sites for several cellular transcription factors immediately upstream of the core promoter. In addition to the aforementioned SP1, a required cofactor for transcriptional initiation [161], binding sites for AP-1, NF-κB, NFAT , USF-1, and COUP-TF are highly conserved within the HIV regulatory subunit [162-165]. The observation that T-cell activation promotes HIV transcriptional suggests that inducible transcription factors are likely to be strong candidates of cellular HIV transactivators [166]. Indeed, AP-1, NFAT, and NF-κB have each been demonstrated to be direct transactivators of HIV-1, and mutation or deletion of their respective binding sites within the provirus impairs viral replicative capacity [164]. Overexpression of these transcription factors induced by T-cell activation drives increased activity of the HIV-1 promoter [167-169]. While it is likely that the induction of each of these transcription factors is involved in the overall effect of T-cell activation on HIV transcription, NF-κB appears to play the most important role [170, 171]. Inhibition of NF-κB strongly ablates

the induction of HIV transcription by T-cell activating stimuli [172], and point mutations within NF-κB binding sites in the HIV provirus reduce viral transcription 100-fold [173]. Given its impact on HIV transcription, NF-κB has been the focus of intensive study.

V NF-κB/Rel Family of Transcription Factors

The NF-κB/Rel family of transcription factors is arguably the most extensively studied collection of eukaryotic transcription factors. NF-κB/Rel is a broadly expressed and evolutionary conserved family of transcription factors with homology to early systems of inducible gene expression observed in arthropods and unicellular eukaryotic organisms, and influences apoptotic, proliferative, inflammatory, and developmental processes. NF-κB was originally described as a B-cell restricted factor that bound to the Ig κ-enhancer [174]. Subsequent investigation demonstrated that NF-κB consists of 65- and 50- kD subunits, termed p65, and p50, which homo- or heterodimerize and bind DNA [175, 176]. After the protein sequence of p65 was determined, substantial homology was recognized with the reticuloendotheliosis virus oncogene, v-rel, and its cellular homolog c-Rel [177]. The molecular cloning of p50 demonstrated further homology between the amino-terminal domains of p50, p65, and v-rel, establishing the rel-homology domain (RHD) and the Rel family [178]. In addition to p65 (RelA), p50 (NF-κB1), and c-Rel, two additional RHD-containing factors have been described, RelB and p52 (NF-κB2) [179-181], completing the mammalian NF-κB family.

The amino-terminal RHD common to each of the Rel family members mediates DNA-binding, nuclear localization, and dimerization, producing a broad range of homo- and heterodimeric NF-κB complexes [177, 178, 182-185]. RelA, RelB, and c-Rel

contain transcriptional activating domains (TADs) in their carboxy-terminal regions [180, 186, 187], a feature absent in p50 and p52. The absence of TADs in this latter group has raised the suggestion that p50 and p52 homodimres may serve as transcriptional repressors [188]. This suggestion is bolstered by the constitutive nuclear accumulation and binding of these complexes to transcriptionally inactive genes [189, 190]. In contrast, TAD-containing NF-κB complexes are largely cytoplasmically localized in the absence of NF-κB-inducing stimulus, and absent from transcriptionally inactive promoters [191].

A family of NF-κB inhibitors, the IκBs, bind to and occlude nuclear localization signals and DNA binding regions present in the RHD of NF-κB dimers [192]. The IκB family includes IκBα, IκBβ, IκBγ,IκBε, IκBζ, Bcl-3, p100, p105, and share homology through an ankryin-repeat domain that mediates intermolecular interactions with NF-κB complexes [193-199]. Despite its similarity to IκBs, Bcl-3 does not inhibit NF-κB activity, but acts instead as a transcriptional activator in concert with p52 homodimers [200]. The carboxy-terminal regions of p105 and p100 are cotranslationally and posttranslationally processed, respectively, by the 26S proteasome to produce p50 and p52 proteins that lack ankyrin repeats [201-203]. In the absence of their inhibitory ankyrin reagion, p50 and p52 continue to bind RelA, RelB, or c-Rel, and enhance DNA binding and nuclear import of these complexes.

The prototypical NF-κB complex, comprised of a heterodimer of RelA and p50, is ubiquitously expressed, despite its original characterization as a B-cell restricted factor. In unstimulated cells, RelA/p50 heterodimers are associated predominantly with IκBα, an association that largely precludes DNA binding and transactivation properties of NF-

κB, and promotes cytoplasmic retention of RelA/p50 complexes through an offset in the rate of nuclear-cytoplasmic shuttling [193, 204]. Induction of cellular activation by T-cell receptor ligation initiates a cascade of phosphorylation events culminating in the phosphorylation of IκBα by the IκB kinase signalsome complex, comprised of IKKγ/NEMO, IKK1, and IKK2 [205-207]. Phosphorylated IκBα is a substrate for polyubiquitylation by E3 ligases, and is subsequently degraded by the 26S proteasome, liberating the RelA/p50 complex [208-210]. Mutation of IκBα serines targeted for phosphorylation by the IKKs produces an inducible degradation resistant transdominant repressor of NF-κB, termed the IκBα super repressor (IκBαSR) [211]. An additional non-canonical pathway of NF-κB is induced upon IKK1 homodimer phosphorylation of p100, which promotes its ubiquitylation and proteolytic removal of the carboxy-terminal IκB-like region, producing an active p52/RelB heterodimer [212]. More recent findings have demonstrated a third IKK-independent pathway of NF-κB activation mediated by RSK1 phosphorylation of RelA, a modification which promotes IκBα disassembly from NF-κB complexes [213]. Regardless of the mechanism of induction, each method of activation induces nuclear accumulation and DNA binding of NF-κB complexes, permitting transactivation of κB-regulated genes.

The NF-κB response to inducing stimulus is subject to remarkably tight temporal control. Nuclear localization of RelA/p50 heterodimers following induction peaks at 30-minutes post-stimulation, and rapidly subsides to uninduced levels within an hour of induction [210]. This "shut-off" of NF-κB transcription factors is a consequence of a negative feedback loop provided by the κB-responsive expression of IκBα. Induction of

NF-κB rapidly promotes expression of IκBα, which binds to and promotes nuclear export of the activated NF-κB complex. The transient nature of the NF-κB response is also impacted by κB-driven expression of A20 [214, 215], an inhibitor of kinase events upstream of the IKK signalsome complex, thus restricting further activation of the IKKs. The IKKs themselves are subject to a degree of autoregulation, and signal-induced activation of IKK2 has been demonstrated to induce autophosphorylation of caboxy-terminal residues that inhibit its kinase activity [216]. How this auto-inhibitory effect is temporally regulated is not yet clear.

Activated NF-κB binds to decameric DNA sequences with a consensus sequence of 5'-GGGRNNYYCC-3', though a substantial degree of degeneracy of this sequence is permitted. This degeneracy is thought to underlie binding site preference of the various NF-κB dimers, and provides a basis for the diversity of observed patterns of NF-κB dependent gene expression. Chromatin immunoprecipitation analysis of NF-κB binding *in vivo* has demonstrated that many κB loci are sequentially bound by multiple NF-κB complexes [217]. TNF-α stimulation of CD4 T-lymphocytes drives rapid association of RelA/p50 with the IL-8 promoter, followed by RelB/p52. It is unclear how this sequential pattern of recruitment shapes the pattern of expression of IL-8 and other genes. Additionally, it is unclear whether one NF-κB species actively displaces another or rather that sequential binding reflects the predominant nuclear species of NF-κB.

Study of NF-κB has focused on RelA and NF-κB1/p50 largely because transgenic mice deficient in these genes exhibit profound developmental and immune deficiencies. RelA-/- mice die *in utero* at embryonic d15-16 due to liver failure caused by TNF-α induced apoptosis of hepatic cells [218]. This observation underscores the contribution

of NF-κB to anti-apoptotic processes. Dual RelA-/- TNF-α receptor -/- mice survive to birth [219], but exhibit a broad array of deficiencies including impaired immune and inflammatory responses marked by an absence of proliferative cellular expansion in response to T- or B-cell activation, defects in dermal differentiation, and more subtle changes in behavior [220, 221]. Taken together, these support a role for RelA as a master regulator of a broad range of biology.

Deletion of p50 [222], though non-lethal, produces profound defects in humoral immunity, and p50-/- B-cells fail to proliferate or produce appropriate antibody responses to bacterial infections. Mice deficient in RelB mature without overt defect aside from splenomegaly and myeloid hyperplasia [223, 224]. T-cell and B-cell development is grossly normal, however $RelB^{-/-}$ B-cell proliferation in response to activating stimuli is limited [225]. Similarly, mice deficient in c-Rel exhibit normal hematopoetic development, however are unresponsive to mitogenic stimuli, and fail to produce IL-2 [226, 227].

Much of NF-κB activity is regulated by its cytoplasmic sequestration mediated by IκBs, however additional control of the pathway is mediated by post-translational modification of the transcription factor complex. NF-κB inducing stimuli drive phosphorylation of RelA, a modification that increases its affinity for DNA *in vitro* [228]. MSK-1 and PKAc mediate phosphorylation of serine 276 in the RHD of RelA. Mutation of this serine residue reduces RelA transactivation of κB-dependant reporters 30-75% [229, 230]. Phosphorylation of RelA at serine 311 induced by PKCζ similarly enhances the overall transcriptional response. Additional RelA kinases and sites of

phosphorylation have been described, including IKK1, IKK2, and RSK1, (reviewed in [231]).

RelA activity is also regulated by acetylation, a reversible modification that dictates the nuclear duration of NF-κB complexes through an unclear mechanism [232]. Acetylation of RelA at lysine 221 enhances DNA binding and impairs assembly with IκBα. Conversely, acetylation at lysine 310 does not appear to affect either IκBα association or DNA binding, but is the most important for transcriptional activity. [233]. Acetylation of K310 is positively regulated by phosphorylation at S276, implying directionality in modification [231]. Whether these modifications activate transcription in a ubiquitous or gene-specific manner is unclear.

Additional modifications of RelA including ubiquitylation have been described, however the biological consequences of these modifications are not yet clear. Other NF-κB family members are post-translationally modified by a similarly diverse range of modifications, however it is unclear whether or how the these modifications contribute to the overall activity of NF-κB-stimulated gene expression.

NF-κB binding to cognate enhancers drives increased transcriptional activity, however, the precise series of molecular events underlying this increase is not completely understood. The TAD in RelA has been demonstrated to associate with a range of histone and polymerase modifying transcriptional coactivators. RelA recruitment of p300, a histone acetyltransferase, is thought to promote hyperacetylation of histones proximal to κB enhancer elements [234], a modification associated with transcriptional activation (see section III) [235]. Histone acetyltransferases P/CAF and CBP are

similarly observed to associate with RelA and may provide functional redundancy, or modulate distinct acetylation events [236].

In addition to promoting local histone acetylation, RelA association with various kinases may promote histone and other transcription factor phosphorylation. A tripartite p300/RelA/cyclin dependent kinase 2 (CDK2) complex has been observed [237], and CDK inhibitors DRB and flavopyridol strongly inhibit NF-κB inducible gene expression. More recent findings support a role for P-TEFb (CDK9/CycT1) as the RelA-associated CDK-containing complex [238]. This latter observation suggests that RelA may be functionally homologous to Tat in the context of HIV-1 expression. In view of the temporal restriction placed on NF-κB by the negative feedback loop, RelA- and Tat-mediated recruitment of P-TEFb to the HIV LTR provides a compellingly complementary model of a source for initial and sustained RNA polymerase II phosphorylation and HIV-1 transcriptional processivity.

Coactivator associated arginine methyltransferase (CARM1), a histone methyltransferase has been observed to associate with RelA-p300 complexes [239], suggesting a role for histone methylation in NF-κB induction of gene expression. Knockdown of CARM1 expression appears to reduce κB-driven gene expression in some contexts, suggesting that methylation could be a positive factor in NF-κB transactivation. Reports of transcriptionally repressive HDAC1 and HDAC2-association with RelA are similarly in conflict with prevailing models of NF-κB transactivation, however knockdown studies of these deactetylases have not been performed [240]. Association of p50-p50 homodimers with HDAC1 has been reported, an association that mediates transcriptional repression of IL-8 expression in the absence of NF-κB activation [190].

In this context, NF-κB activation serves a two-fold effect: displacement of an actively repressive p50-p50-HDAC1 complex with a positively acting RelA-p50-p300-CDK complex. Further exploration of NF-κB interactions with histones will be fundamental to increased understanding of the molecular underpinnings of NF-κB transactivation. A direct aim of this study was to examine the modulation of basal transcription factor modificaton and histone acetylation by NF-κB in the context of the latent HIV-1 promoter (Chapter 3).

VI The J-Lat Model of Post-Integration HIV-1 Latency

The rarity of latently infected cells, and the absence of any discernable marker of HIV-1 latency greatly hamper efforts to directly study mechanism in the most relevant pool. Additionally, the obfuscating pool of defective integrated proviruses precludes meaningful interpretation of data derived from bulk cultures of patient blood. Nonetheless, cultured outgrowth of virus from HIV-1-infected patient blood offers the most relevant target of study. This method of assay requires patient cooperation, costly cell-sorting and culturing techniques, and lengthy procedure. Taken together with the low level of cellular targets isolated from each patient, experiments conducted in this system must chosen with great economy. Thus, culture models of HIV-1 latency must be employed to identify lead candidate mechanisms of this treatment-resistant form of infection.

Suggestions of latent HIV-1 infection of human T-cell lines had been observed as early as 1986, with 5-iodo2'-deoxyuridine inducible expression of virus from A3.01-derived ACH-2 cells observed [241]. Similarly, cytokine-inducible HIV-1 was observed

in a cultured U937 monocyte cell line, U1 [242]. Several NF-κB inducing-stimuli, including UV-light, PMA, LPS, and TNF-α were subsequently demonstrated to be strong inducers of HIV–1 from apparently latently infected cell lines [243-246]. By contrast, latently infected THP-1 monocytes are unresponsive to NF-κB-inducing stimuli, but strongly responsive to 5-azacytidine, an inhibitor of DNA-methylation, suggesting the possibility that multiple mechanisms underlie HIV-1 latency [247].

Efforts to understand the molecular mechanisms driving HIV latency within culture systems focused initially on aberrant patterns of RNA expression common to several models [248]. Mutation in the HIV-1 splicing factor Rev or its RRE cognate RNA binding site was proposed as a source of latency [249], as was alteration of the viral transactivators Tat or of its target TAR RNA site [166]. Reconstitution of various latently infected cell clones demonstrated that wt Rev and Tat supplied in *trans* could rescue viral production in many cell lines [250]. The failure of these manipulations to recover infectious virus from ACH-2 cells suggested that the locus of proviral integration could be an important determinant of HIV-1 latency [251]. However, subsequent analyses demonstrated that the TAR region contained an inactivating mutation, thus discarding the notion that the position of proviral integration is responsible for this form of HIV-1 latency [112]. HIV-1 latency in U1 cells proved to be a consequence of an inactivating mutation in Tat.

Proviral mutation does not account for all HIV-1 latency observed in tissue culture models. A mutational basis for the HIV-1 latency observed in OM10.1 [252], a HL-60 promyelocytic cell line, and J1.1 [253], a Jurkat T-cell line, has not yet been determined, however the rarity of early HIV-1 mRNA transcripts observed in these cell

lines supports a failure in transcriptional initiation, likely due to integration locus-specific positional silencing [254]. Indeed, the site of proviral integration has been shown to dramatically influence basal HIV-1 transcription rates [116]. It is unclear whether latent HIV-1 infections are immediately established, or whether there is some transition through a state of viral production. CEM T-cell lines chronically infected with HIV-1 eventually shutdown viral production and reproducibly establish latent states, suggesting that actively infected cells are capable of regressing into a latent state [255, 256]. Determination of which of these various mechanisms is responsible for HIV-1 latency *in vivo* has been difficult to ascertain, due to the restrictions of studying primary sources of latency.

Criticism of most models of HIV-1 latency hinges on the fact that single loci of integration are studied, thus observations made in one model may be reflective of a particular integration locus and not generalizable. Efforts to escape this criticism have produced systems more reflective of *in vivo* biology, including the SCID-HU model developed by Brooks and Zack [104]. In this model, mice reconstituted with a human immune system through thymic transplantation are infected with an HIV virus expressing a marker gene in place of viral *env*. A single-round of infection of CD4+ cells undergoing thymopoesis occurs, thus allowing a brief window of activation during which early proviral reverse transcription and genomic integration can complete. This model shares some of the difficulties associated with patient blood culture, in that only a fraction of cells harbor latent HIV-1 provirus, and analyses must be done in bulk culture with an interfering backdrop of defective integrated virus. In contrast, manipulations intended to effect the latent HIV-1 pool can be performed *in vivo*, allowing for broader

assessment of activating approaches. However, due to the overall expense of animal models and their inherent difficulty of manipulation, this system is not ideal for the dissection of specific molecular mechanisms underlying HIV-1 latency.

To improve upon previous models of HIV-1 latency, Jordan and Verdin infected Jurkat T-cells with an HIV provirus at low multiplicity of infection engineered to express Aequoria Victoria green fluorescent protein (GFP) in place of the viral Nef gene, thus allowing rapid and inexpensive fluorescent assessment of viral gene expression [117]. This virus additionally contained a frameshift in *env* to restrict infection to a single round. The resulting infected cells were screened for inducible GFP expression, and a multitude of latently-infected Jurkat T-cell clones, or J-Lat, were derived. A similar approach has been used to generate latently HIV-1 infected monocytes [257]. Subsequent analysis revealed that the inducible, latent nature of the HIV-1 provirus in many J-Lat clones was not a result of cellular or proviral mutation, but rather likely a consequence of cis-acting transcriptionally repressive factors surrounding the viral integration site. The isolation of multiple clones exhibiting similar biology despite separate integration loci allows for the assessment of generalizability of findings across clones. As such, the J-Lat model presents the most authentic and easily monitored model of HIV-1 latency to date, and as such has been a key component of the studies presented herein.

Studies of J-Lat cell clones have revealed that DNA-methylation, long thought to be a major determinant of basal HIV transcriptional activity, is not associated with latent HIV-1 proviruses [122]. Analysis of integration locus demonstrated that transcriptionally silent HIV-1 proviruses often reside within actively transcribed genes [118], an apparent paradox. Fluorescent analysis of unstimulated J-Lat cells reveals that the majority of cells

express no GFP, indicating an absence of HIV-1 gene expression. Some background level of GFP-expressing cells is observed in each clone, ranging from 0.1% to 10% of the total population. Stimulation of these cells with various NF-κB-inducing stimuli induces a subset of cells to express GFP, typically ranging from 20%-80% depending on the J-Lat clone studied. Generally, J-Lat clones that have a higher fraction of GFP-positive cells when unstimulated will also have a higher fraction when stimulated. Similar fractional responses in GFP-expression have been observed in cell lines derived by other groups with similar strategies. It is unclear why a clonally derived cell-line should differentially respond to stimulus.

An aim of this work was to further understand the variegated nature of HIV-1 induction within the clonal J-Lat model. Additionally, a major aim was to explore the molecular mechanisms underlying HIV-1 latency within the J-Lat cell lines, with particular focus on the role of the NF-κB pathway. We found that latency in the J-Lat model is largely a consequence of a failure of transcriptional initiation. This failure is regulated NF-κB1/p50 recruitment of HDAC1, a complex that promotes histone deacetylation, chromatin compaction, and exclusion of the RNA polymerase II complex. We further discovered efficient induction of latent HIV-1 gene expression requires sustained induction of NF-κB. This is a consequence of rapid rerepression of transcriptional initiation in absence of HIV-activating stimulus. The findings of this work point to unexpected intersections of NF-κB and HIV-1 latency, and further underscore the key role of chromatin in transcriptional regulation.

Figure 1

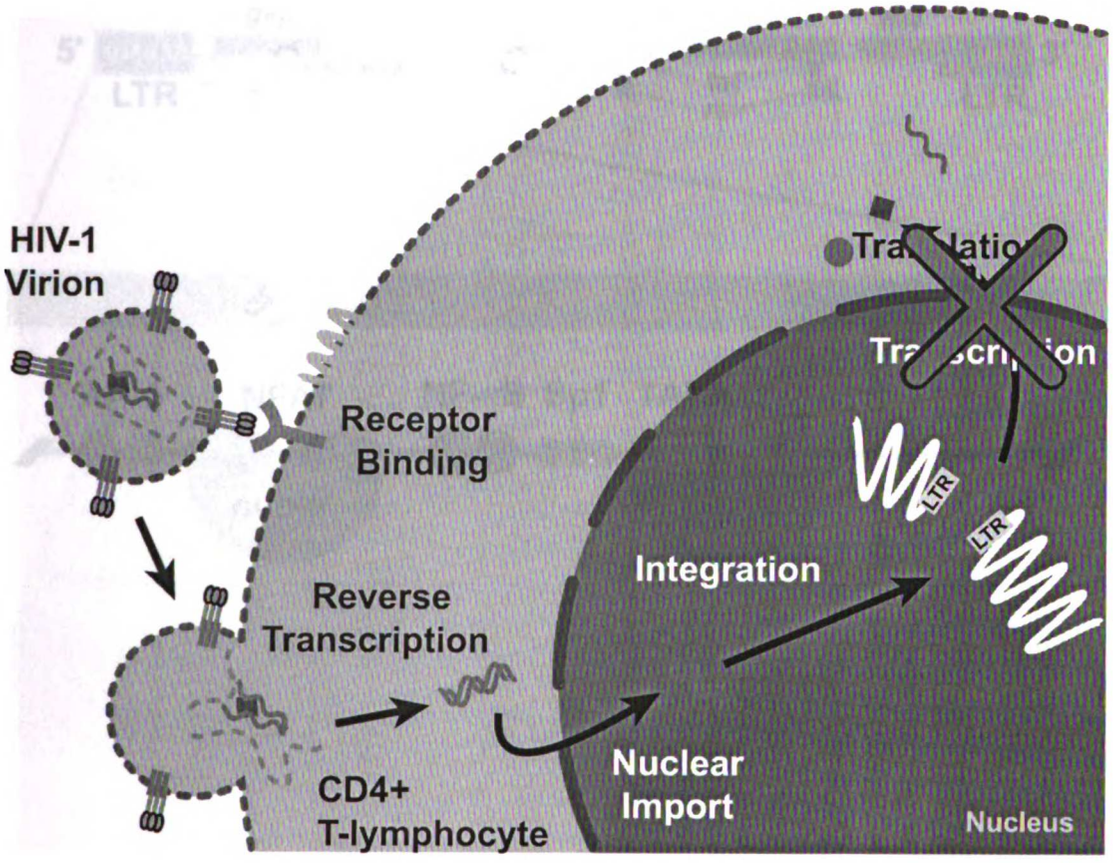
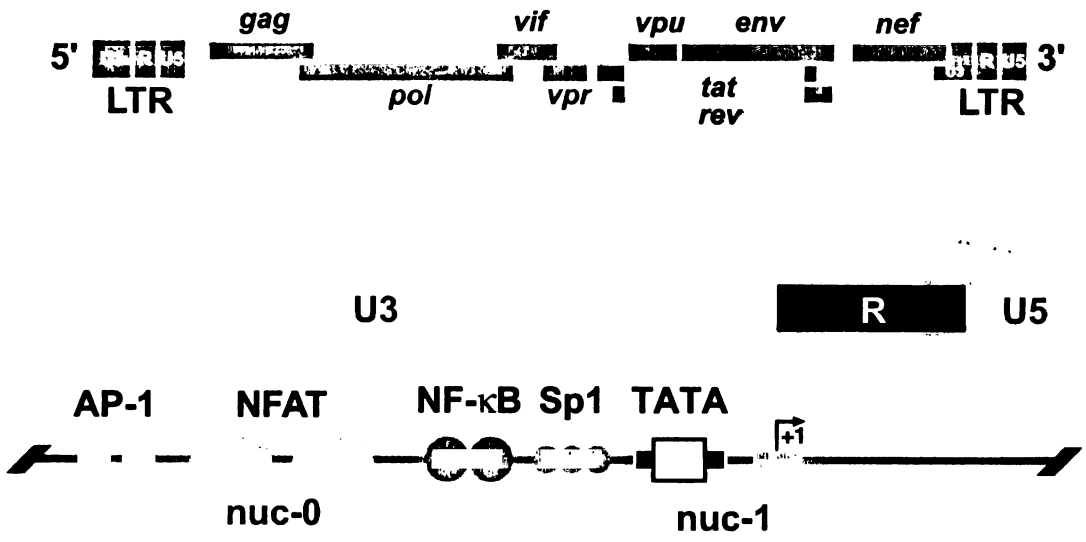


Figure 2


5'
LTR
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NFAT
NF-κB
Sp1
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+1
nuc-0
nuc-1

Figure 3

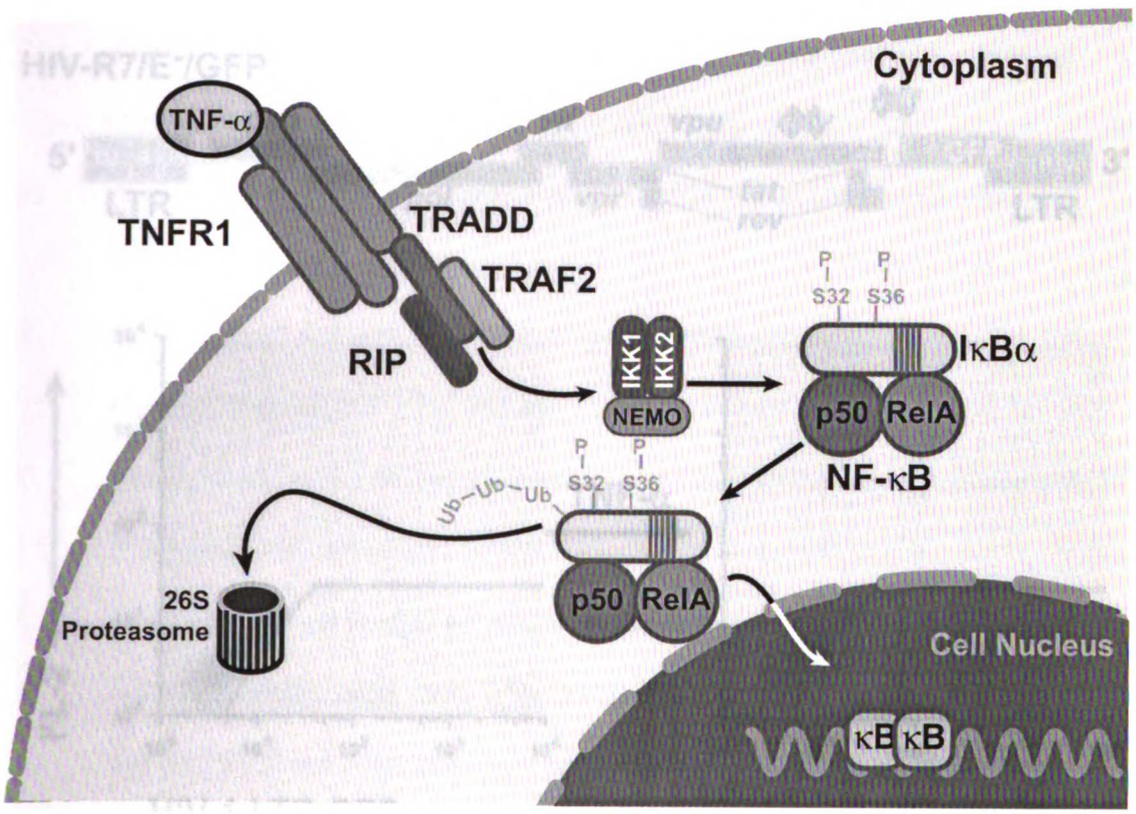
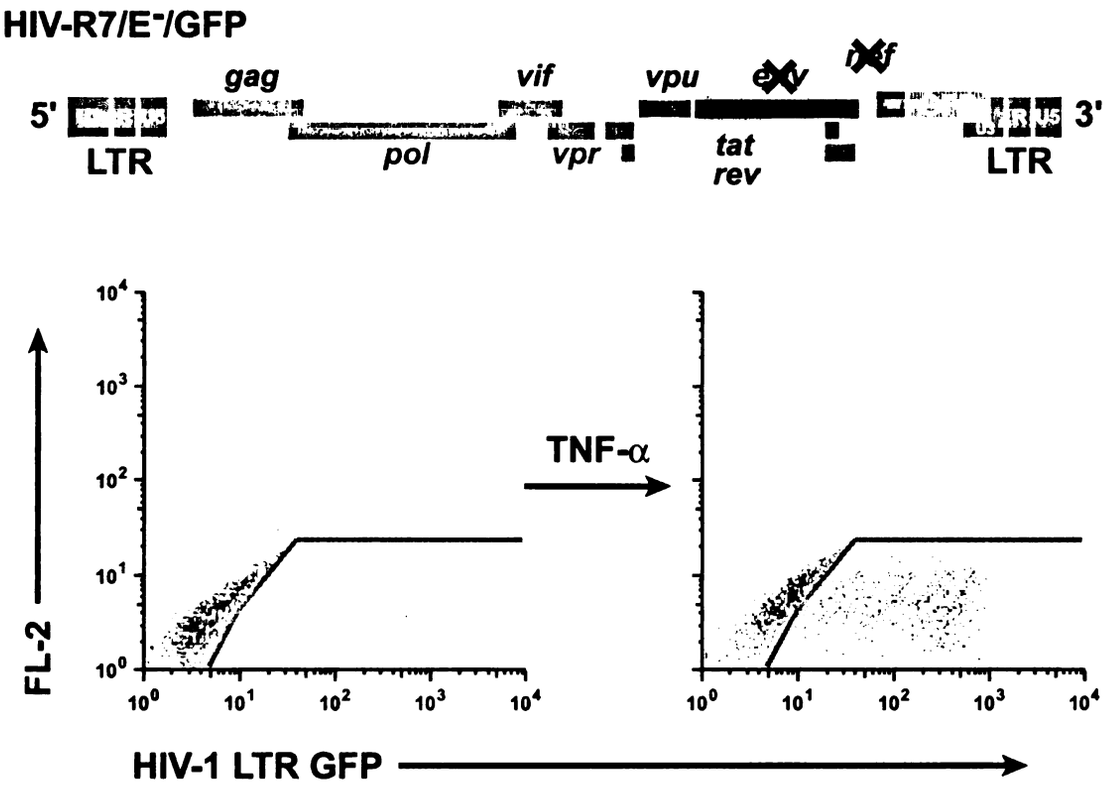


Figure 4



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Chapter 2

Prostratin antagonizes HIV latency by activating NF-κB

Introduction

Early efforts to purge the pool of latent HIV-1 infection from patients focused on broadly targeted immune activating therapies. Studies of the T-cell receptor-activating antibody OKT3 and the general inflammatory cytokine IL-2 as adjuvant therapies to HAART demonstrated that these broad approaches were ineffective [1, 2]. These failures prompted a search for new candidate HIV-1 latency antagonists, including traditional Eastern medicines in addition to more established Western pharmaceuticals.

Prostratin, 12-deoxyphorbol 13-acetate, is a phorbol ester that forms the therapeutic basis of Somoan traditional medicines used in treatment of a wide spectrum of ailments. Derived from the bark of *homanthalas nutans*, prostratin bears strong molecular similarity to phorbol myristate acetate (PMA), an established mitogenic T-cell activator, and a strong inducer of HIV-1 transcriptional activity [3]. In addition to its mitogenic properties, PMA is a known tumor-promoter, thus precluding its use as a therapeutic in human subjects. In contrast, prostratin exhibits no such carcinogenic properties at concentrations in 100-fold excess of tumorigenic doses of PMA [4]. As such, further exploration of the prostratin as an antagonist of HIV-1 latency was warranted.

Examination of prostratin in U1 and ACH-2-based models of HIV-1 latency confirmed that the phorbol ester is capable of enhancing the transcriptional activity of integrated HIV provirus [5, 6]. These studies, however, were performed in models of latency that are consequences of viral mutation in HIV-1 *tat* or TAR, and thus are incomplete models of HIV-1 postintegration latency [7, 8].

To extend and expand upon previous studies of prostratin antagonism of HIV-1 latency, we assessed the effect of the non-tumor promoting phorbol ester on multiple J-Lat cell clones. Additionally, we examined the mechanism of prostratin activity, isolating the induction of the NF-κB family of transcription factors as a necessary determinant of prostratin induction of latent HIV-1. The intersection of NF-κB activation was demonstrated to be direct, as chromatin immunoprecipitation assays revealed prostratin-induced binding of the strong NF-κB transactivators RelA to the κB elements present in the HIV-1 enhancer of the latent provirus. Further characterization of upstream events identified prostratin induction of several novel, classical, and atypical PKC-family members. Targeted interference with these subclasses suggested that the novel PKCs are of central importance in prostratin induction of HIV-1 latency. Additionally, the observation of dissimilar patterns of PKC membrane translocation suggested a mechanistic basis for the non-tumor-promoting effect of prostratin vs. its oncogenic cousin PMA.

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Prostratin Antagonizes HIV Latency by Activating NF-κB*

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A subset of quiescent memory CD4 T cells harboring integrated but transcriptionally silent proviruses poses a currently insurmountable barrier to the eradication of the human immunodeficiency virus (HIV) in infected patients. Induction of HIV gene expression in these latently infected cells by immune activating agents has been proposed as one approach to confer sensitivity to antiretroviral therapy. Interest has recently focused on the non-tumor-promoting phorbol ester, prostratin, as a potential agent to activate latent HIV proviruses. Using multiple Jurkat T cell lines containing integrated but transcriptionally latent HIV proviruses (J-Lat cells), we now demonstrate that prostratin effectively activates HIV gene expression in these latently infected cells. We further show that prostratin acts by stimulating IKK-dependent phosphorylation and degradation of IκBα, leading to the rapid nuclear translocation of NF-κB and activation of the HIV-1 long terminal repeat in a κB enhancer-dependent manner. In contrast, NFAT and AP-1 are not induced by prostratin. Using chromatin immunoprecipitation assays to identify host transcription factors recruited to the latent HIV-1 promoter in living cells, we find that prostratin induces RelA binding. Analysis of potential upstream signal transducers demonstrates that prostratin stimulates membrane translocation of classical, novel, and atypical protein kinase C (PKC) isoforms. Studies with isoform-specific PKC inhibitors suggest that the novel PKCs play a particularly prominent role in the prostratin response. These findings provide new insights into the molecular pathway through which prostratin antagonizes HIV latency highlighting a central role for the action of NF-κB.

A small but clinically important fraction of CD4$^+$ memory T cells in HIV-1 [1]-infected patients contain integrated but transcriptionally inactive proviruses (1). These latently infected cells retain the ability to produce infectious virus following cellular activation by specific antigen or various cytokines (2). The half-life of these CD4$^+$ memory T cells is at least 44 months; consequently, elimination of this viral reservoir is projected to require administration of antiretroviral therapy (ART) for at least 60 years (3, 4). Accordingly, the rebound viremia routinely observed in patients terminating effective ART probably involves viral reseeding from this long-lived latent reservoir. Indeed, studies examining the genetic characteristics of rebound virus have found substantial similarities between virus present in the latent pool and that emerging after cessation of therapy (5). New approaches to the elimination of these latently infected cells are urgently needed (6).

One proposed strategy to eliminate this pool of latently infected cells is to purge the virus by activating the CD4$^+$ memory lymphocytes in concert with ART administration (7). However, clinical efforts aimed at eliminating the pool of latently infected cells by induction with IL-2 or anti-CD3 OKT3 antibodies have yielded discouraging results (8, 9).

The failure of initial attempts to deplete the latently infected pool of CD4 T cells reflects in part our limited understanding of the molecular mechanisms governing HIV latency. HIV latency is characterized by transcriptional inactivity, but the underlying cause of this inactivity remains unknown and may be multifactorial. The HIV long terminal repeat (LTR) is a well-characterized transcription regulatory element that contains DNA-binding domains for a broad range of transcription factors, including AP-1, NFAT, Sp1, and NF-κB (see Ref. 10 for a review). NF-κB and Sp1 have been demonstrated to be key factors in the stimulation of HIV replication, and viruses lacking binding sites for either factor display attenuated replicative capacity (11). The absence of active NF-κB in the nuclei of quiescent memory CD4 lymphocytes could play a key role in promoting proviral latency in this lymphocyte subset.

Study of the latently infected reservoir *in vivo* is greatly hampered by the fact that infected patients may contain only 10^5 to 10^6 latently infected CD4 memory T cells and that in their latent state these cells are virtually impossible to distinguish from uninfected CD4 memory T cells (12). We have employed a recently developed Jurkat T-cell model of postintegration HIV latency termed J-Lat (13). J-Lat T-cell clones are infected with full-length HIV proviruses and contain the *Aequorea victoria* green fluorescent protein (GFP) gene in lieu of Nef, thus permitting epifluorescence monitoring of viral transcriptional activity. Under basal conditions, little or no GFP expression is detected; however, transcriptional activation of the latent provirus leads to GFP expression, which can be detected at the single-cell level by flow cytometry. In contrast to other previously studied models of HIV latency where mutations have been detected in the HIV Tat gene or TAR element, the J-Lat cells contain wild-type Tat and TAR and appear to be highly representative of the latently infected cells present *in vivo*. Because multiple latently infected clones have been independently established, clone- and integration locus-specific phenotypes can be excluded.

Recent interest has focused on prostratin, a nontumorigenic phorbol ester, as a potential antagonist of HIV latency (14, 15).

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[1] The abbreviations used are: HIV-1, human immunodeficiency virus, type 1; ART, antiretroviral therapy; LTR, long terminal repeat; GFP, green fluorescent protein; FACS, fluorescence-activated cell sorting; TNF, tumor necrosis factor; PMA, 4-α-phorbol 12-myristate 13-acetate; PBMC, peripheral blood mononuclear cell; PKC, protein kinase C.

Prostratin is derived from the Samoan plant *Homalanthus nutans* and has been used in traditional medicine to treat yellow fever and other conditions (16, 17). Early studies of prostratin demonstrated that this agent inhibits HIV replication *in vitro* at micromolar concentrations via down-regulation of the cellular HIV receptors CD4 and CXCR4 (14, 18). More recent studies have shown that prostratin also promotes transcriptional activation of latent HIV proviruses in latently infected thymocytes present in the SCID-hu Thy/Liv model (15). Prostratin may also not elicit the broader and less desirable changes in cellular activation associated with IL-2 and OKT3 therapies (15) and thus may be clinically useful. Currently, the precise molecular basis of prostratin antagonism of HIV latency is unknown. We now describe a series of studies dissecting the mechanism of prostratin induction of latent HIV provirus in J-Lat cells.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions—J-Lat clones 6.3, 8.4, 9.2, and 10.6 and sorted peripheral blood lymphocytes were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin, streptomycin, and L-glutamine. Cells were stimulated with 0.1–10 μM prostratin (LC Laboratories), 10 ng/ml TNF-α (R&D Systems), or 10 μM 4-α-phorbol 12-myristate 13-acetate (PMA) in the presence or absence of 1 μM ionomycin (Sigma) for 8 or 14 h as indicated. For PKC inhibition studies, cells were incubated with 10–1000 nM Go6983, 5 μM Go6976 (Calbiochem), 5 μM Go6850 (Biomol), or 100 nM TER14687 (Sigma) for 1 h prior to cellular activation.

Transient Transfection and Luciferase Assays—J-Lat 6.3 or 9.2 cells were expanded to 0.5 to 1 × 10^6 cells/ml and resuspended in serum-free RPMI at 4 × 10^7 cells/ml. 15 μg of expression vector DNA was added (adjusted with pCMV4 carrier DNA as necessary) to 0.4-ml aliquots of the cell suspension followed by electroporation (250 V, 950 microfarads in 0.4-cm cuvettes; Invitrogen) and resuspension in 6 ml of complete RPMI. Reporter plasmids containing the luciferase gene positioned immediately downstream of the full-length, ΔκB, ΔAP-1, or ΔSp1 LTR of HIV-1 or transcription cassettes containing tandem copies of the NFAT, NF-κB, AP-1, or Sp1 enhancers. These reporter plasmids were cotransfected with 0.1 μg of *Renilla* luciferase expression vector (Promega) to monitor differences in transfection efficiency. 15–20 h after electroporation, cells were stimulated as indicated for 5 h at 37 °C. The dual luciferase assay system (Promega) was employed to measure resultant luciferase activity using a Wallac microbeta 1450 luminometer.

Flow Cytometry Analysis and FACS—J-Lat 6.3 or 9.2 cells were transfected with 2 μg of pMACS-Kk (H2Kk) and 13 μg of empty pCMV4 or pCMV4-IκBαSR expression vector DNA 48 h prior to stimulation. Transfected and nontransfected cells were stimulated as indicated for 14 h at 37 °C. Following incubation, cells were stained with biotin-anti-H2Kk antibody, washed, and stained with streptavidin-APC (Pharmingen). Cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson). For intracellular anti-Gag immunostaining, J-Lat 9.2 cells were fixed in 4.7% paraformaldehyde and stained with anti-p24 RD1 (Coulter) antibody in buffer containing 0.1% Triton for permeabilization. Samples were washed once and resuspended in PBS prior to analysis. Compensation was performed with monostained cultures. Data were analyzed with FloJo software (Treesoft). For FACS sorting experiments, J-Lat 9.2 cells were incubated with 2 μM prostratin or 10 ng/ml TNF-α for 24 h, washed twice in complete medium, and incubated for an additional 24 h prior to FACS sorting on a FACS DIVA cell sorter. GFP-negative cells were collected and incubated in complete medium for 3 days, followed by incubation with either prostratin or TNF-α for 24 h. GFP expression was assessed by flow cytometry.

Immunoblotting Analysis—J-Lat 6.3 or 9.2 cells were adjusted to 1 × 10^6 cells/ml and stimulated with TNF-α or prostratin for various times. Cells were then lysed on ice in egg lysis buffer (50 mM HEPES, pH 7, 250 mM NaCl, 1% Nonidet P-40, 5 mM EDTA) for 20 min and clarified by microcentrifugation. Lysates were next added to an equal volume of 2× Laemmli buffer (25 mM Tris, 200 mM glycine, 0.1% SDS) and heated to 95 °C for 5 min. Proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and immunoblotted with various antibodies.

In Vitro Kinase Assay—J-Lat 6.3 or 9.2 cells were adjusted to 2 × 10^6 per ml in serum-free medium and stimulated with 10 μM prostratin, 10 ng/ml TNF-α, or 10 μM PMA for various times. Cells were lysed in egg lysis buffer and immunoprecipitated with anti-NEMO antibodies to isolate IKK-containing signalsomes as previously described (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) (19). *In vitro* kinase assays were performed as described utilizing glutathione *S*-transferase-IκBα-(1–62) as an added exogenous substrate. Reactions were terminated by the addition of an equal volume of 2× Laemmli buffer. Reaction products were separated by SDS-PAGE, electrophoretically transferred to a polyvinylidene difluoride membrane, and exposed to HyperFilm at −80 °C. Following autoradiography, membranes were probed with anti-IKK1 antibodies (Santa Cruz Biotechnology) to determine the amounts of kinase immunoprecipitated in each sample.

Electrophoretic Mobility Shift Assay—J-Lat 6.3 or 9.2 cells or sorted peripheral blood lymphocytes were adjusted to 2 × 10^6 cells/ml and stimulated with 10 ng/ml TNF-α or 2 μM prostratin for various times. After stimulation, nuclear extracts were prepared as previously described (20). For analysis of κB enhancer binding proteins, 2.5 μl of the nuclear extracts were incubated with poly(dI-dC) and salmon sperm DNA for 10 min, followed by the addition of [γ-^{32}P]ATP-labeled consensus κB or Oct-1 enhancer oligonucleotides (Promega). For supershift analysis, 2 μl of anti-p65 or anti-Hck polyclonal antibodies (Santa Cruz Biotechnology) or anti-p50/p105 antiserum were added prior to the addition of radiolabeled oligonucleotides. Samples were separated on nondenaturing gels and analyzed by autoradiography.

Chromatin Immunoprecipitation Assay—J-Lat 6.3 and 9.2 T cells were adjusted to 1 × 10^7 cells/ml and stimulated with 10 ng/ml TNF-α or 2 μM prostratin for 30 or 45 min, respectively. Cells were incubated in 1% formaldehyde for 10 min at room temperature, followed by termination of the reaction with 125 mM glycine. Cells were washed twice in PBS and three times in run-on lysis buffer (10 mM Tris-Cl, pH 7.6, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40) and resuspended in micrococcal nuclease reaction buffer (10 mM Tris-Cl, pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM $CaCl_2$, 4% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride). Lysates were digested with 100 units of micrococcal nuclease for 10 min at 37 °C, and the reaction was stopped with 3 mM EGTA. DNA was then sheared by sonication to achieve an average size of 0.5–1 kb. Extracts were clarified by centrifugation at 13,000 rpm for 10 min. For immunoprecipitations, 5 μl of anti-RelA polyclonal antibody (Santa Cruz Biotechnology) were mixed with 600 μl of extract for 2 h, followed by the addition of agarose protein A beads for 2 h. A nonspecific agarose protein A bead binding control performed in the absence of added antibody was included with each sample. Following incubation, beads were washed exhaustively, and proteins were eluted in elution buffer (25 mM Tris-Cl, pH 7.5, 10 mM EDTA, 0.5% SDS) with a 15-min incubation at 60 °C. Eluates were digested with Pronase (Sigma) and decross-linked by incubation at 65 °C for 12 h. DNA was isolated by phenol/chloroform extraction, and PCR analysis was performed. For detection of the HIV-1 LTR, DNA oligonucleotide primers LTRκB-primer5 (5′-AGGTTTGACAGCCGCCTA-3′) and LTRκBprimer3 (5′-AGAGACCCAGTACAGGCAAAA-3′) specific for a 203-bp region encompassing the κB binding sites in the HIV LTR were used for PCR amplification. For detection of control β-actin DNA, two primers β-actin5 (5′-GTCGACAACGGCTCCGGC-3′) and β-actin3 (5′-GGTGTGGTGCCAGATTTTCT-3′) specific for a 239-bp region in the β-actin gene were used. 35 cycles of amplification were performed using *Taq* polymerase (Qiagen), and the products were analyzed on a 2.5% agarose gel. Images were acquired using an EagleEye digital camera.

PKC Translocation Assay—J-Lat 6.3 cells were adjusted to 1 × 10^6 cells/ml and incubated with either 10 μM prostratin, 10 μM PMA, or 10 ng/ml TNF-α for 7 or 30 min. After stimulation, cells were washed in PBS and lysed in hypotonic lysis buffer (10 mM Hepes, 10 mM KCl, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) for 5 min on ice, followed by freezing in liquid nitrogen. Lysates were thawed and centrifuged at 14,000 rpm, and cytosolic supernatant was collected. Remaining lysate pellet was resuspended in Nonidet P-40 buffer (20 mM Tris, pH 7.5, 200 mM NaCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride) and centrifuged at 14,000 rpm, and membrane extract supernatant was collected. Cytosolic and membrane extract preparations were separated by SDS-PAGE, electrophoretically transferred to polyvinylidene difluoride membranes, and probed with various isotype-specific anti-PKC antibodies (BD Biosciences).

Isolation of Primary CD45RO$^+$ CD4$^+$ T-lymphocytes—Primary peripheral blood mononuclear cells (PBMCs) were separated from erythrocytes by Ficoll density gradient centrifugation. CD4$^+$ cells were isolated from PBMCs by negative selection bead sorting (Miltenyi). CD45RO$^+$ T cells were subsequently sorted from this pool by FACS. >99% purity of CD45RO$^+$, CD4$^+$, and CD3$^+$ sorted cells was confirmed by immunofluorescent staining and flow cytometric analysis.

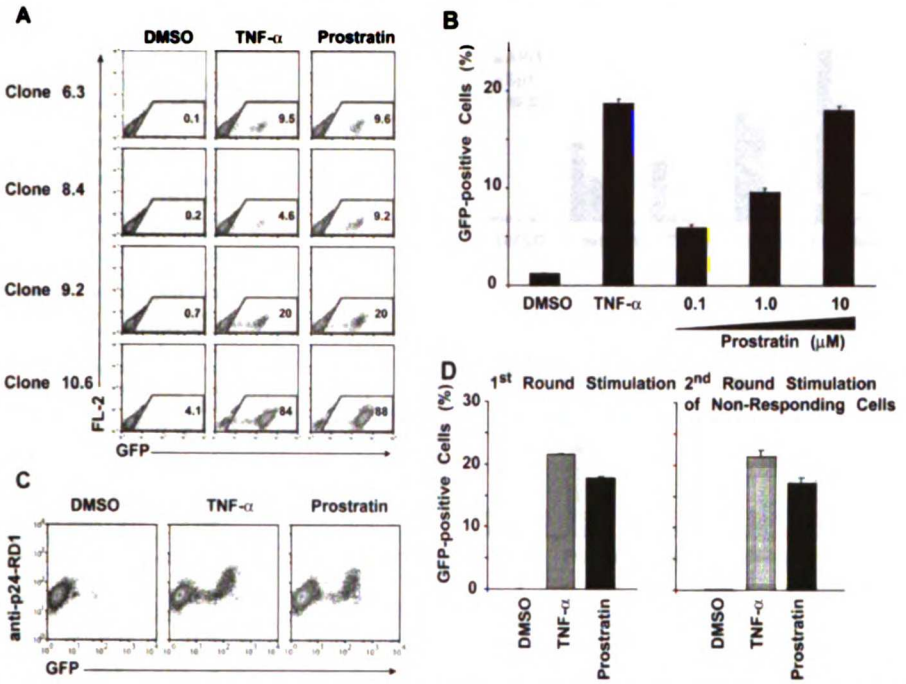


FIG. 1. **Prostratin antagonizes HIV latency in J-Lat T-lymphocytes.** *A*, prostratin activates latent proviruses present in multiple J-Lat clones. J-Lat clones 6.3, 8.4, 9.2, and 10.6 were stimulated with 10 μM prostratin (*right column*) or with TNF-α (*middle column*) or with medium containing 0.1% Me_2SO (*DMSO; left column*). GFP expression reflecting transcriptional activation of the latent HIV proviruses was measured by flow cytometry. The percentage of cells activated is presented in the GFP-positive gate. Note that prostratin and TNF-α activate a similar response, although only a fraction of the cells respond. *B*, prostratin antagonizes HIV latency in a dose-dependent manner. J-Lat clone 9.2 cells were stimulated with TNF-α or 0.1–10 μM prostratin. The *error bars* indicate S.D. *C*, prostratin induction of GFP expression is correlated with intracellular expression of HIV Gag. J-Lat clone 9.2 cells were stimulated with medium containing carrier Me_2SO, TNF-α, or 10 μM prostratin and stained for intracellular Gag expression with anti-p24 monoclonal antibodies conjugated to phycoerythrin (*bottom row*). GFP expression is plotted on the *x* axis, and intracellular anti-p24-phycoerythrin immunofluorescence on the *y* axis. The staining pattern obtained indicates that the majority of GFP-positive cells also express the HIV late gene product Gag. A similar response was observed in other J-Lat clones. *D*, prostratin and TNF-α induce a variegated response in J-Lat T cells. Stimulated J-Lat 9.2 cells, which failed to express GFP in response to either prostratin or TNF-α, were isolated by FACS and subjected to a second round of prostratin or TNF-α stimulation. Levels of GFP expression induced in the initially nonresponsive cells were measured by flow cytometry. Note that levels of GFP expression induced during the second round of stimulation in initially nonresponding cells are similar to that induced in previously unstimulated, nonsorted cells.

RESULTS

Prostratin Induces HIV Gene Transcription in Latently Infected J-Lat T Cells—To examine the effect of prostratin in a lymphocyte-based model of HIV postintegration latency, J-Lat clones 6.3, 8.4, 9.2, and 10.6 were incubated for 14 h with 10 μM prostratin, 10 ng/ml TNF-α, or 0.1% Me_2SO as a buffer control. In the absence of stimulation, J-Lat 6.3, 8.4, and 9.2 cells expressed virtually no GFP, whereas J-Lat 10.6 cells were ~4% positive, indicating low-level HIV gene expression (Fig. 1*A*, *left column*). Following treatment with prostratin, GFP expression was induced to various and characteristic degrees in each of the four J-Lat T cell lines (Fig. 1*A*, *right column*). Of note, a higher proportion of J-Lat 10.6 cells displayed GFP epifluorescence following prostratin stimulation. TNF-α activated GFP expression in each of the latently infected cell lines to levels comparable with that induced by prostratin. Short term (<24-h) prostratin treatment induced little or no toxicity as assessed by the forward and side light scattering properties of the treated cells (data not shown). Prolonged exposure to prostratin (>2 days), however, induced substantial growth arrest and cell death at concentrations greater than 500 nM (data not shown). To more precisely define the dose-response relationship for prostratin, J-Lat 9.2 cells were stimulated with 0.1–10 μM concentrations of the agonist. Prostratin induced latent HIV LTR-driven expression of GFP at concentrations as low as 0.1 μM, with minimal toxicity (Fig. 1*B*). When concentrations of prostratin higher than 10 μM were tested, increased short-term toxicity was observed (data not shown).

Because the GFP gene is substituted for the early expressed Nef gene of HIV-1 in the J-Lat model, we investigated whether prostratin also activated HIV late gene expression. Following 14 h of stimulation with prostratin or TNF-α, immunostaining for intracellular Gag expression revealed expression of this Rev-dependent late gene product in a majority of the GFP positive subset of cells (Fig. 1*C*). Together, these studies demonstrate that prostratin induces dose-dependent transcriptional activation of the latent HIV provirus present in four independently derived J-Lat cell lines. Further, prostratin stimulation leads to expression of both early and late HIV gene products. However, at high doses or after prolonged exposure, prostratin induces cytotoxic effects.

Both prostratin and TNF-α induce GFP expression in only a subset of the J-Lat cells. Given that the J-Lat cell lines are clonal, the nonuniform response of these lines to activating stimuli suggested a variegated response. However, it was unclear whether the unresponsive cells were permanently inactivated or able to undergo activation in a second round of stimulation. To distinguish between these possibilities, J-Lat 9.2 cells were incubated

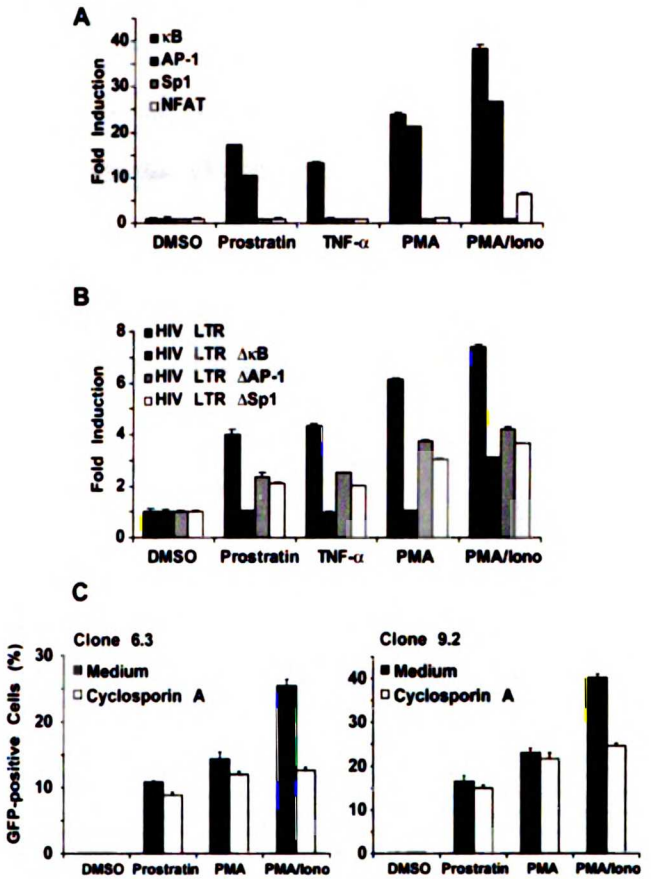


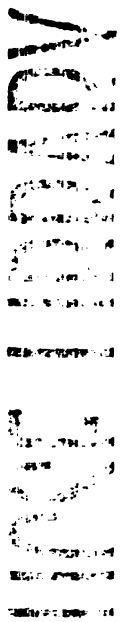
FIG. 2. **Prostratin activates the HIV-1 LTR through induction of NF-κB.** *A*, prostratin activates κB- and AP-1 luciferase reporter plasmids. J-Lat 9.2 cells were transfected with κB-, AP-1-, Sp1-, or NFAT-luciferase reporter plasmid DNA and stimulated with 2 μM prostratin, TNF-α, PMA, or a combination of PMA and ionomycin. Luciferase activity was measured 5 h later. The *error bars* indicate S.D. Note that prostratin activates the κB and AP-1 reporter plasmids but not the Sp1 or NFAT reporter plasmids. Similar results were obtained in J-Lat 6.3 cells. *B*, an HIV LTR lacking the κB enhancers is not induced by prostratin. J-Lat 9.2 cells were transfected with HIV1-LTR-, HIV1-LTRΔκB-, HIV1-LTRΔAP-1-, and HIV1-LTRΔSp1-luciferase reporter plasmid DNA and 14 h later were stimulated with 2 μM prostratin, TNF-α, PMA, or PMA/ionomycin. Luciferase activity was measured after 5 h of stimulation. The *error bars* depict S.D. Note the lack of prostratin activation of the HIV1-LTRΔκB reporter plasmid. Similar results were obtained in J-Lat 6.3 cells. *C*, prostratin induction of the HIV LTR is not inhibited by cyclosporin A. J-Lat 6.3 or 9.2 cells were preincubated with cyclosporin A for 1 h followed by stimulation with 2 μM prostratin, TNF-α, PMA, or PMA/ionomycin. Note that the prostratin response is not inhibited by cyclosporin A, whereas the PMA/ionomycin response is partially impaired, suggesting a component of NFAT-mediated stimulation under these conditions of activation.

with either prostratin or TNF-α, and the initially unresponsive cells were purified and stimulated in a second round with prostratin or TNF-α. In parallel, previously unstimulated, unsorted J-Lat 9.2 cells were stimulated with prostratin or TNF-α. The second round of either prostratin or TNF-α stimulation induced GFP expression in ~20% of originally nonresponsive cells, a fraction that was equivalent to that observed in the previously unstimulated cells (Fig. 1*D*). These findings indicate that an increasingly greater proportion of the entire J-Lat cell population is activated by serial stimulation, with the fractional response during each cycle remaining constant.

Prostratin Activation of the HIV LTR Is Mediated through NF-κB—We next explored the signaling pathway activated by prostratin in J-Lat cells that mediates activation of the latent HIV proviruses. The HIV-1 LTR contains binding sites for several inducible transcription factors, including NF-κB, NFAT, AP-1, and Sp1 (10). To assess the effects of prostratin on the induction of these transcription factors, J-Lat 9.2 cells were transfected with NF-κB-, AP-1-, Sp1-, or NFAT-luciferase reporter plasmids and stimulated with either 2 μM prostratin, 10 ng/ml TNF-α, 10 μM PMA, or combinations of PMA and 1 μM ionomycin. Prostratin activated the NF-κB- and AP-1-responsive reporter constructs but failed to activate the Sp1 or NFAT luciferase reporters (Fig. 2*A*). The magnitude of the NF-κB response to prostratin was less than that obtained with PMA either added alone or in combination with ionomycin but was comparable with TNF-α.

To assess more directly the role of NF-κB/Rel factors in prostratin activation of the HIV LTR, J-Lat 9.2 cells were transfected with luciferase reporter plasmids containing either the wild type HIV-1 LTR, the LTR lacking the two κB enhancers, the LTR lacking the AP-1 enhancers, or the LTR lacking the Sp1 enhancers. Prostratin induced 4-fold stimulation of the HIV-LTR-Luc reporter relative to unstimulated controls (Fig. 2*B*) but failed to activate the HIV-LTRΔκB-Luc reporter. However, co-incubation with PMA and ionomycin stimulated luciferase activity with this ΔκB reporter, indicating the induction of a non-NF-κB/Rel transcription factor with LTR-activating properties. Additionally, prostratin induced ~2.5-fold stimulation of the HIV-LTRΔAP-1 and HIV-LTRΔSp1 reporters, indicating that neither AP-1 nor Sp1 is required for HIV LTR responsiveness to prostratin. Together, these findings support a central role for NF-κB/Rel induction in prostratin-mediated activation of the latent HIV LTR and exclude a necessary role of AP-1 and Sp1.

Because NFAT has been implicated in the activation of the LTR involving a site overlapping the κB sites (21), we further tested the potential involvement of NFAT as a mediator of prostratin antagonism of HIV latency by treating cells with cyclosporin A. This agent blocks the activation of NFAT by antagonizing calcineurin-mediated dephosphorylation of NFAT (22). J-Lat 6.3 or 9.2 cells were preincubated with cyclosporin A or with medium containing comparable quantities of Me_2SO used to dissolve cyclosporin A and then stimulated



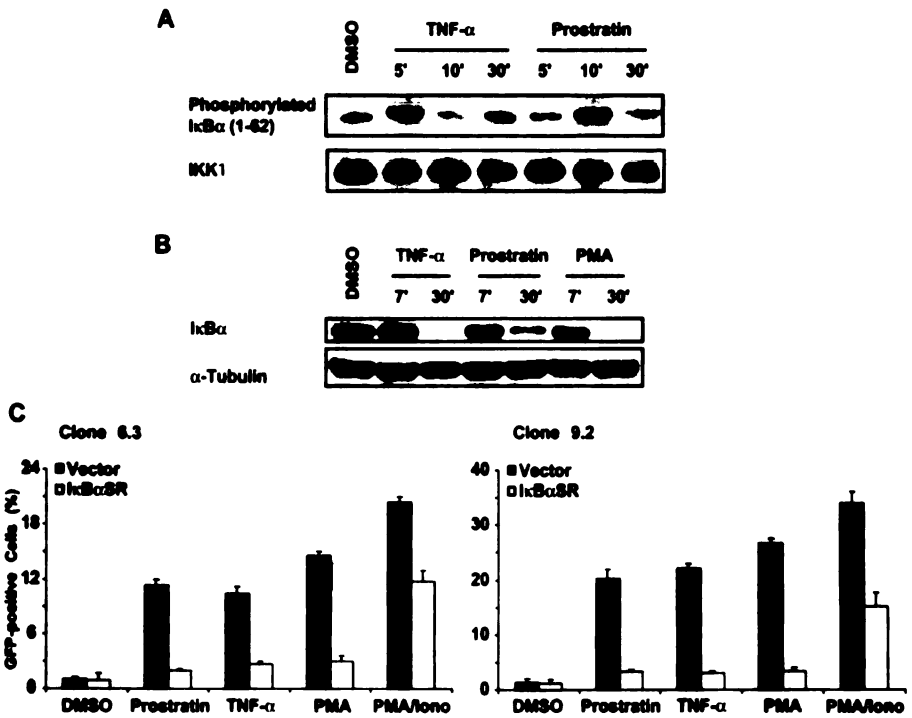


FIG. 3. **Prostratin activation of the latent HIV LTR involves IKK activation and degradation of IκBα.** *A*, prostratin stimulates activation of endogenous IKKs. J-Lat 6.3 cells were stimulated with TNF-α or 2 μM prostratin for 5, 10, or 30 min, followed by immunoprecipitation of IKK complexes with anti-IKKγ antibodies. These complexes were analyzed for enzymatic activity in *in vitro* kinase assays utilizing glutathione *S*-transferase-IκBα-(1–62) as an exogenous substrate. Levels of IKK1 immunoprecipitated under each condition are shown in the *lower panel*. Note that both TNF-α and prostratin activate the IKK complexes, although the prostratin response occurs with slightly slower kinetics. Similar results were obtained other J-Lat clones. *B*, prostratin induces IκBα degradation. J-Lat 6.3 cells were stimulated with TNF-α or 2 μM prostratin for 10, 15, or 30 min, and cellular lysates were immunoblotted with antibodies specific for IκBα or β-tubulin. Similar results were obtained with other J-Lat clones. *C*, IκBα super repressor (IκBαSR) inhibits prostratin activation of latent HIV provirus. J-Lat 6.3 or 9.2 cells were transfected with expression vectors encoding either an IκBαSR protein expression vector or vector alone, in combination with an H2Kk marker of transfection, followed by stimulation with 2 μM prostratin, TNF-α, PMA, or PMA/ionomycin. H2Kk-expressing cells were analyzed by flow cytometry for GFP expression. Note that the IκBαSR markedly inhibits prostratin-mediated activation of the latent provirus present in both J-Lat clones but only partially inhibits the response elicited by PMA and ionomycin.

with prostratin, TNF-α, PMA, or combinations of PMA and ionomycin for 14 h, followed by assessment of GFP expression. Prostratin, TNF-α, and PMA each induced strong GFP responses, and these responses were not impaired by the addition of cyclosporin A (Fig. 2*C*). Conversely, combined stimulation of these cells with PMA and ionomycin induced higher levels of GFP expression than observed with PMA alone, and this response was partially inhibited by cyclosporin A. These findings suggest that NFAT induction plays a role in the activation of latent HIV provirus in J-Lat cells stimulated with PMA and ionomycin. The cyclosporin-resistant component of HIV proviral activation observed in these cells probably reflects the induction and action of NF-κB/Rel factors. These findings support a central role for NF-κB/Rel induction in prostratin antagonism of HIV latency.

Prostratin Induction of NF-κB Is Associated with IKK Activation—Among the initial events in the classical signaling cascade leading to NF-κB induction is the activation of IKK kinase activity (see Ref. 23 for a review). To assess whether the macromolecular IKK complex is stimulated by prostratin, we assessed IKK enzymatic activity in J-Lat 6.3 cells in *in vitro* kinase assays. After stimulation of J-Lat cells with either prostratin or TNF-α, endogenous IKK complexes were immunoprecipitated from cell lysates using an anti-NEMO antibody. IKK enzymatic activity was detected by phosphorylation of glutathione *S*-transferase-IκBα-(1–62) added as an exogenous substrate. Prostratin markedly stimulated IKK kinase activity after 10 min, with relatively little activity observed at 5 or 30 min of stimulation (Fig. 3*A*). In contrast, TNF-α stimulation led to a more rapid increase in IKK activity, peaking at 5 min and returning to base line at 10 and 30 min. These findings suggest that prostratin induction of NF-κB is mediated through activation of the IKKs and that the kinetics of this response are slightly slower than that elicited by TNF-α.

Activated IKKs target the IκBα inhibitor of NF-κB for phosphorylation on serines 32 and 36, leading in turn to the rapid ubiquitylation and degradation of the inhibitor by the 26 S proteasome (24). To assess the functional consequence of prostratin induction of IKK kinase activity, we assessed the ability of prostratin to induce IκBα degradation in J-Lat 6.3 cells. Prostratin, like TNF-α and PMA, induced degradation of IκBα (Fig. 3*B*). However, consistent with the observed delay in IKK activation, the degradation of IκBα induced by prostratin at 30 min was less complete than that induced by TNF-α.

To further evaluate whether IκBα degradation plays a key role in the prostratin response, J-Lat 6.3 or 9.2 cells were transfected with expression vectors encoding a nondegradable mutant of IκBα (IκBα SS32/36, termed the IκBα super repressor, or IκBαSR) or with vector alone, along with a mouse MHC H2Kk surface antigen transfection marker plasmid (Fig. 3*C*).

Cell surface immunofluorescence staining for H2Kk permitted rapid identification of the transfected subset of cells. Following transfection and culture for 24 h, cells were stimulated with prostratin, TNF-α, PMA, or a combination of PMA and ionomycin for 14 h. Cells expressing H2Kk were then analyzed for GFP expression indicative of latent HIV provirus activation. Prostratin, TNF-α, PMA, and PMA/ionomycin effectively induced latent HIV LTR-mediated GFP expression in the H2Kk-transfected cells. In contrast, cells transfected with IκBαSR failed to increase GFP expression in response to prostratin, TNF-α, or PMA. Consistent with dual induction of NF-κB and NFAT by combination of PMA and ionomycin, GFP expression in dually treated cells was only partially inhibited by IκBαSR. These findings provide further evidence that NF-κB activation plays an important role in prostratin-mediated activation of latent HIV proviruses in the J-Lat cells.

Prostratin Induces NF-κB Nuclear Translocation and DNA Binding—Following the degradation of IκBα, the liberated NF-κB transcription factors translocate into the nucleus and engage cognate κB enhancers. To assess whether prostratin stimulation provided sufficient stimulus for RelA nuclear translocation and DNA binding, we assessed κB enhancer DNA binding activity in nuclear extracts from J-Lat 6.3 cells stimulated with prostratin or TNF-α for 30 or 45 min. Extracts were incubated with [γ-^{32}P] radiolabeled κB enhancer oligonucleotides. Parallel assays were performed with γ-^{32}P-labeled Oct-1 DNA oligonucleotides to assess loading and integrity of the nuclear extracts. Unstimulated nuclear extracts contained κB enhancer binding activity consistent in migration with p50-p50 homodimers (Fig. 4*A*). Following prostratin and TNF-α stimulation, a more slowly migrating complex was observed. Based on antibody supershifting, this complex corresponds to the prototypical NF-κB complex of p50-RelA heterodimers bound to the κB enhancer (data not shown). Consistent with the observed slower kinetics of both IKK activation and IκBα degradation, prostratin induction of NF-κB DNA binding occurred somewhat slower than the response elicited by TNF-α.

Prostratin Induces Direct RelA Binding to the Integrated HIV LTR—NF-κB could either directly activate the LTR of the latent proviruses present in J-Lat cells by binding to the κB enhancer or indirectly by inducing the expression of a second gene product, which in turn drives LTR transcription. To investigate whether RelA is directly recruited to the HIV LTR *in vivo* following prostratin stimulation, chromatin immunoprecipitation assays were performed. J-Lat clones 6.3 and 9.2 were treated with TNF-α or prostratin for 30 or 45 min, respectively. The longer time course for prostratin was selected in view of its slower kinetics of induction. Following stimulation, cells were cross-linked, and DNA was fragmented by micrococcal nuclease digestion and sonication. Lysates were immunoprecipitated with anti-RelA or anti-Sp1 antibodies and agarose A beads or with agarose A beads alone, and DNA-protein cross-links were reversed. Coimmunoprecipitated DNA was isolated and input, RelA-immunoprecipitated, and agarose A control samples were probed by PCR for HIV LTR κB binding site DNA and for downstream HIV and β-actin sequences as nonspecific controls. In the absence of stimulation, samples immunoprecipitated with anti-RelA amplified low to undetectable levels of HIV LTR κB binding site DNA (Fig. 4*B*). Following stimulation with TNF-α or prostratin, anti-RelA immunoprecipitated samples from both J-Lat clones 6.3 and 9.2 amplified significant quantities of HIV LTR DNA. Importantly, these samples did not amplify β-actin negative controls beyond background levels, demonstrating specificity of the DNA immunoprecipitation. In contrast, Sp1 was constitutively bound to the latent HIV LTR, with no observable change in occupancy occurring in response

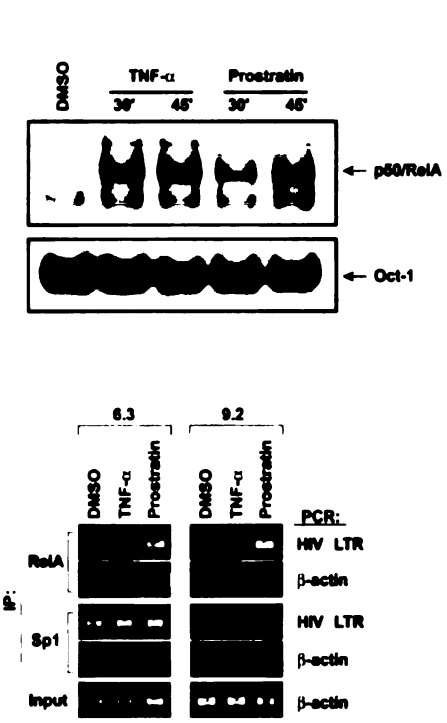


FIG. 4. **Prostratin stimulates nuclear NF-κB DNA binding and induces RelA-mediated recruitment to the HIV-1 LTR *in vivo*.** *A*, prostratin induces NF-κB DNA binding. J-Lat 6.3 cells were stimulated with TNF-α or prostratin for the indicated times. Nuclear extracts were isolated and subjected to electrophoretic mobility shift assay with γ-^{32}P-labeled κB enhancer DNA probes. Oct-1 binding was studied in parallel as a control for loading and integrity of various protein extracts. *B*, prostratin induces RelA recruitment to the latent HIV-1 LTR *in vivo*. J-Lat clones 6.3 or 9.2 were stimulated with TNF-α or 2 μM prostratin for 30 or 45 min, respectively. Chromatin immunoprecipitation assays were performed using anti-RelA or anti-Sp1 antibodies and probed for the HIV LTR DNA sequences spanning the κB enhancer or for nonspecific control β-actin. Note that prostratin and TNF-α stimulation induced RelA recruitment to the κB enhancer of the HIV-1 LTR in both latently infected J-Lat clones, whereas Sp1 is constitutively bound and unaffected by either treatment. *DMSO*, Me_2SO; *IP*, immunoprecipitation.

to TNF-α or prostratin stimulation. These findings support a model of prostratin action involving the induction and direct recruitment of NF-κB to the latent HIV LTR.

Prostratin Activation of the Latent LTR Requires Activity of Novel PKC Isoforms—Previous studies of prostratin induced down-regulation of CD4 and CXCR4 identified a PKC-dependent signaling step (18). However, it remained unclear whether single or multiple PKC isoforms were involved in this response. To characterize the profile of PKC isoforms induced by prostratin, we prepared cytoplasmic and membrane fractions from J-Lat 6.3 cells treated with prostratin, TNF-α, or PMA and analyzed which PKC isoforms were induced to translocate from the cytoplasm to the membrane. Such translocation serves as an early marker of PKC activation (25). Prostratin induced rapid translocation of PKC-θ and -β from the cytoplasm to the membrane fraction, with similar efficiency to PMA (Fig. 5*A*). Membrane translocation of PKC-α, -γ, -δ, and -ζ was also induced by prostratin; however, this translocation response was incomplete and greatly reduced in comparison with the response induced by PMA. In contrast, TNF-α did not induce membrane translocation of any of these PKC isoforms. Immu-

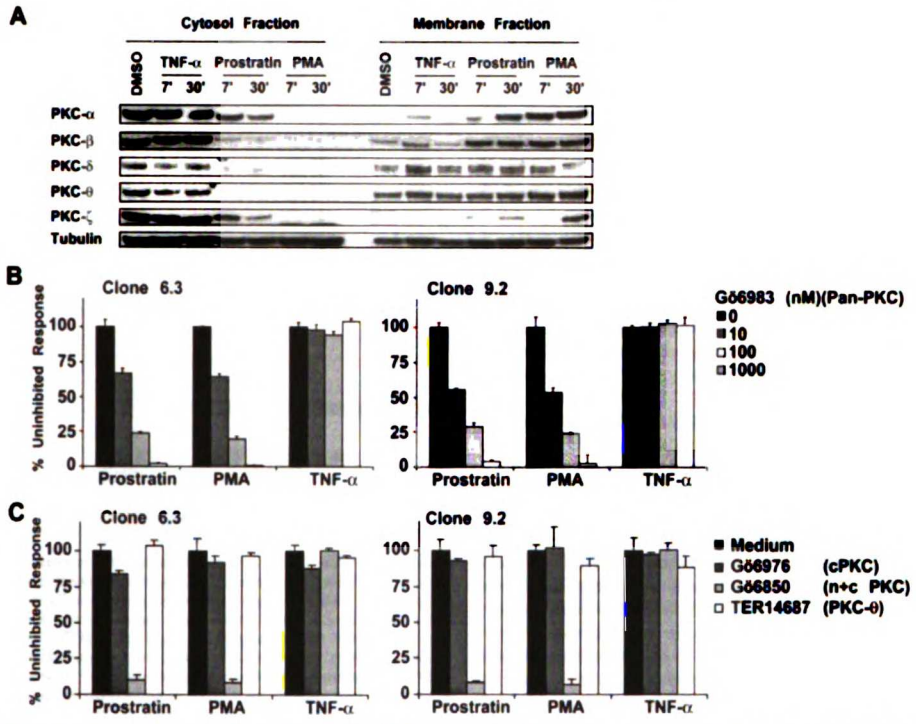


FIG. 5. **PKC activation and prostratin-mediated stimulation of latent HIV provirus.** *A*, prostratin induces membrane translocation of multiple PKC isoforms. J-Lat 6.3 cells were treated with Me_2SO (*DMSO*), TNF-α, 2 μM prostratin, or PMA for the indicated times. Cytoplasmic and membrane extracts were prepared, and proteins were separated by SDS-PAGE followed by immunoblotting with antibodies specific for various PKC isoforms. β-Tubulin was employed as a loading control for these extracts. Similar responses were observed in other J-Lat clones. *B*, PKCs participate in prostratin antagonism of HIV latency. J-Lat clones were incubated with a pan-PKC inhibitor, Gö6986, prior to stimulation with 2 μM prostratin, PMA, or TNF-α. Results for J-Lat 6.3 and 9.2 cells are shown. The percentage inhibition of total GFP expression was measured by flow cytometry. Note the inhibition of the prostratin and PMA responses with Gö6983 but no effect of this inhibitor on the TNF-α induced response. *C*, novel PKCs probably play a role in prostratin-mediated activation of latency. J-Lat 6.3 or 9.2 cells were incubated with medium, Gö6976 (an inhibitor of conventional PKC isoforms only), Gö6850 (an inhibitor of novel and conventional PKC isoforms), or TER14687 (an inhibitor the novel PKC isoform PKC-θ). Cells were stimulated with 2 μM prostratin, TNF-α, or PMA. The percentage inhibition of total GFP expression was measured by flow cytometry. Note that an inhibitor of conventional PKC isoforms failed to impair the prostratin or PMA response, whereas the inhibitor of conventional and novel PKC isoforms effectively inhibited these responses, and the inhibitor of PKC-θ failed to inhibit prostratin induction of GFP expression.

noblotting for PKC-ε, -ι, -η, -γ, and -λ failed to produce specific signals (data not shown).

To further assess the PKC dependence of prostratin-mediated antagonism of HIV latency, we pretreated J-Lat 6.3 or 9.2 cells with medium or the pan-PKC small molecule inhibitor Gö6983 (26), followed by stimulation with prostratin, TNF-α, or PMA (Fig. 5*B*). Gö6983 strongly inhibited GFP expression induced by both PMA and prostratin, further implicating a PKC-dependent signaling step in this response. In contrast, TNF-α induction of the latent HIV provirus was unaffected by Gö6983, excluding nonspecific toxic effects of this drug. The PKC family of protein kinases includes the classical, novel, and atypical subclasses (see Ref. 27 for review). To investigate whether a specific subclass of PKC mediates prostratin antagonism of HIV latency, we employed PKC subclass-specific inhibitors (Fig. 5*C*). The Gö6850 inhibitor impairs the action of only the conventional and novel PKC isoforms (28). This inhibitor exerts little effect on the atypical ζ, τ, λ, and μ isoforms. In contrast, the Gö6976 inhibitor inhibits the action of only the conventional PKC isoforms, α, β, and γ (28). Preincubation with Gö6850 reduced GFP expression in response to both prostratin and PMA with similar efficiency, indicating that conventional and/or novel subclasses of PKCs are involved. In contrast, the addition of Gö6976 had little effect on prostratin and PMA induction of GFP expression, arguing that prostratin antagonism of HIV latency is not dependent on the conventional subclass of PKC isoforms. Neither inhibitor reduced TNF-α induction of the latent genome. Taken together, these findings suggest that one or more members of the novel PKC subfamily (δ, ε, η, and θ isoforms) play an important signaling role in prostratin activation of the latent HIV-1 LTR.

The novel isoform PKC-θ has been implicated as an important regulator of NF-κB activity during T-cell activation (29–31). To more specifically assess the role of PKC-θ in prostratin induction of the latent HIV LTR, we employed a PKC-θ isoform-specific inhibitor, TER14687 (32). J-Lat 6.3 or 9.2 cells were pretreated with standard medium or with TER14687, followed by stimulation with prostratin, PMA, or TNF-α. In the presence of TER14687, no appreciable inhibition of LTR induction by prostratin relative to untreated samples was observed (Fig. 5*C*, *white bars*). High concentrations of TER14687 proved toxic, precluding the examination of effects at higher doses (data not shown). These findings suggest that either PKC-θ does not play a major role in the prostratin response or that other novel PKC isoforms function in a redundant manner with PKC-θ.

Prostratin Activates NF-κB and Induces NF-κB-dependent Gene Expression in Primary Memory CD4 Lymphocytes—The

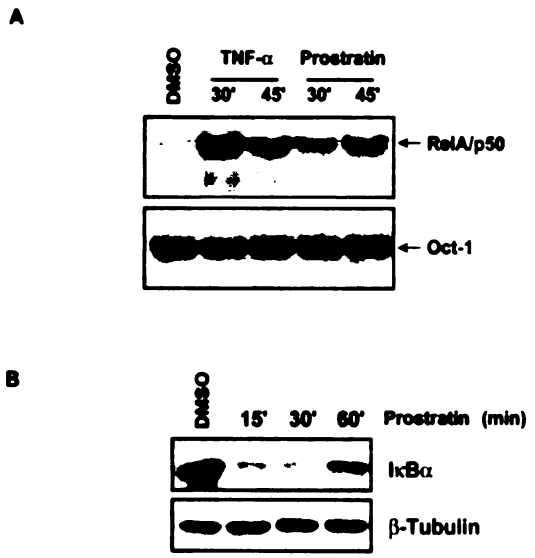


FIG. 6. **Prostratin activates NF-κB and induces NF-κB-dependent genes in primary resting CD4⁺ memory T cells.** *A*, prostratin induces NF-κB DNA binding in primary memory CD4+ lymphocytes. CD45RO⁺ CD4⁺ lymphocytes were sorted from patient PBMCs and stimulated with TNF-α or 2 μM prostratin for the indicated times. Nuclear extracts were prepared and subjected to electrophoretic mobility shift assay with γ-^{32}P-labeled κB enhancer DNA probe. Oct-1 binding was studied in parallel as a control for loading and integrity of various protein extracts. *B*, prostratin induces activation of a NF-κB-responsive gene in primary memory CD4⁺ lymphocytes. Sorted patient PBMCs were stimulated with 2 μM prostratin for the indicated times and cellular lysates were immunoblotted with antibodies specific for the NF-κB-responsive gene, IκBα, or β-tubulin as a control. Note the resynthesis of IκBα at 60 min indicating prostratin activation of functional nuclear NF-κB complexes in these primary cells. *DMSO*, Me_2SO.

latent viral reservoir present in infected patients has been identified within quiescent nondividing CD4⁺ memory T cells arrested in the G_0 phase of the cell cycle. To assess whether our observations within the continuously dividing J-Lat clones reflect the biology of this arrested pool of cells, we sorted CD45RO⁺ CD4⁺ T-lymphocytes from patient blood. This isolated population was confirmed to be G_0-arrested by DNA staining (data not shown). We assessed the inducibility of NF-κB within this pool using electrophoretic mobility shift assay. Sorted patient cells were stimulated with TNF-α or prostratin for 30 or 45 min, and nuclear extracts were prepared and incubated with γ-^{32}P-radiolabeled consensus κB or Oct-1 oligonucleotides. Both TNF-α and prostratin treatment induced a slowly migrating complex indicative of NF-κB activation (Fig. 6*A*). As observed in J-Lat clones, the peak in NF-κB binding activity induced by prostratin was delayed relative to the TNF-α response.

To ensure that NF-κB activation in primary memory CD4⁺ lymphocytes results in NF-κB-dependent gene expression, we assessed the resynthesis of the NF-κB-responsive gene, IκBα. Sorted primary CD45RO⁺ CD4⁺ T-lymphocytes were untreated or treated with prostratin for 15, 30, or 60 min, and cellular lysates were prepared and immunoblotted for IκBα or β-tubulin, the latter serving as a loading control. Prostratin induced complete degradation of IκBα within 30 min of stimulation, consistent with activation of the NF-κB signaling pathway (Fig. 6*B*). Resynthesis of IκBα was observed after 60 min of prostratin stimulation, indicating effective induction of this NF-κB-responsive gene. Taken together, the observed activation of NF-κB and induction of NF-κB-responsive genes in these nondividing primary CD45RO⁺ CD4⁺ T-lymphocytes recapitulates the response observed in the proliferating J-Lat model.

DISCUSSION

In this study, we have explored the mechanism by which the non-tumor-promoting phorbol ester, prostratin, antagonizes HIV latency. Considerable interest has recently focused on prostratin as an *in vivo* agent to purge latent HIV proviruses (6, 14, 15). As a cellular model of HIV latency, we employed the J-Lat T cells, which contain a full-length latent HIV provirus. This provirus contains GFP in place of Nef; thus, transcriptional activation of the latent provirus can be readily detected in individual cells by flow cytometry. We find that prostratin effectively activates the latent provirus in multiple independently derived J-Lat T-cell clones. These results are in agreement with recently published results demonstrating prostratin-mediated activation of latent virus in the SCID-HU Thy/Liv system and in the blood of patients with HAART-suppressed HIV (14, 15). We observed that prostratin induces similar levels of proviral gene transcription as TNF-α, although the kinetics of the prostratin-mediated response are slower. Intriguingly, neither prostratin nor TNF-α induced proviral reactivation in the entire clonal population of latently infected cells. Indeed, it appears that activation of the latent HIV LTR occurs in a variegated manner, a hallmark of epigenetic regulation. When initially nonresponsive cells were restimulated with prostratin or TNF-α, a similar fraction of previously unresponsive cells were induced to express GFP. The underlying mechanism of this fractional responsiveness may lie in heterochromatin epigenesis or alternatively could reflect selective responsiveness of cells at a particular stage in the cell cycle. These findings suggest that the reversal of HIV latency will probably require repeated administration of agonists. Thus, to be clinically useful, such inducers must exhibit relatively low toxicity, permitting patients to withstand long-term, multiround treatments (33).

Prostratin was observed to lack toxicity when applied for short time courses; however, it induced substantial growth arrest and cell death if administered in a concentration of >500 nM for greater than 2 days. If prostratin is to be considered as a human therapeutic, it is unlikely that high-dose or protracted treatment will be tolerated. Consequently, short-term, low-dose treatments will probably be the only alternative, since sustained administration will probably result in dramatically negative side effects.

To delineate the molecular basis for prostratin-mediated activation of the latent HIV LTR, we first examined the ability of prostratin to activate various signaling pathways using reporter plasmids containing κB, AP-1, Sp1, or NFAT enhancer cassettes. We observed that prostratin effectively activated the κB- and AP-1-luciferase reporters but displayed no stimulatory effects on the Sp1 or NFAT reporter constructs. Consistent with a central role for NF-κB stimulation, prostratin failed to induce a mutated version of the HIV LTR lacking the tandem κB enhancers, whereas it promoted expression of AP-1- and Sp1-deficient LTR constructs. These findings are consistent with the prior observation that prostratin induces up-regulation of a number of NF-κB-responsive genes, including IL-1β, IL-8, and osteoprotogerin (14). The observed induction of AP-1 by prostratin is probably reflective of the phorbol ester nature of this compound. It is possible that in addition to the NF-κB and AP-1 pathways, prostratin also stimulates other signaling cascades that either modulate the NF-κB response or function independently. The fact that the profile of gene expression induced by prostratin fails to completely overlap with that found with classical agonists of NF-κB like TNF-α strengthens this possibility.

Further investigation of the pathway of NF-κB activation stimulated by prostratin revealed that prostratin activates the IKKs residing in the macromolecular signalsome complex. Notably, this response occurs with somewhat delayed kinetics compared with the response elicited by TNF-α. We further found that prostratin induces phosphorylation and degradation of IκBα and that expression of a degradation-resistant mutant of IκBα (IκBα super repressor) inhibits prostratin activation of the latent HIV LTR. These findings indicate that the classical NF-κB signaling pathway is stimulated by prostratin.

NFAT has also been proposed to function as a key transcriptional activator of the HIV-1 LTR (34, 35); however, the NFAT inhibitor cyclosporin A failed to impair prostratin-mediated activation of the latent HIV LTR. Of note, latent provirus was activated in a greater fraction of cells when agonists that stimulated both the NFAT and NF-κB pathways were employed. This observation is consistent with early studies demonstrating cooperative induction of the HIV LTR by NF-κB and NFAT (36). These results suggest that strategies to activate latent HIV proviruses involving the induction of both NF-κB and NFAT merit further investigation. Such approaches might also help mitigate the problem posed by the variegated nature of latent HIV provirus activation.

Since the NF-κB pathway is activated by prostratin, and HIV-1 LTR constructs lacking the κB enhancers are not stimulated by prostratin, it was important to confirm that Rel proteins were directly recruited to the HIV-1 LTR *in vivo* following prostratin stimulation. Using chromatin immunoprecipitation assays to study this question, we observed that prostratin stimulation promoted rapid recruitment of RelA to the HIV LTR. In contrast, we observed that Sp1 is constitutively associated with the latent HIV promoter. These findings argue for a direct role of RelA in the prostratin-induced transcriptional response leading to activation of the latent HIV LTR in cells stimulated with either prostratin or TNF-α. Additionally, these observations demonstrate that Sp1 binding is insufficient to promote transcriptional activation of the latent HIV LTR. In the setting of the HIV LTR, RelA synergizes with Sp1 to induce gene expression, probably involving chromatin remodeling steps (37). In the case of the A20 gene, Sp1 independently recruits TFIID to the site of transcriptional initiation (38). Nonetheless, RelA is required to promote efficient transcriptional elongation by the RNA polymerase II complex, probably through its ability to recruit the pTEFb complex that phosphorylates the C-terminal heptapeptide repeat of RNA polymerase II. Indeed, in the absence of Sp1, RelA is probably less efficient in driving LTR transcription (39). Determining the full range of transcription factors bound to the transcriptionally repressed and activated LTR *in vivo* will be instrumental in enhancing our understanding of the molecular events that govern HIV latency. Additionally, analyses of the impact of transcription factor binding on localized chromatin structure may reveal insights into the molecular underpinnings of HIV latency. Such insights may also suggest additional strategies for activation of the latent LTR in a greater fraction of the cells.

Additional events occurring during the activation of NF-κB by prostratin may play an important role in the transcriptional activation of the latent HIV LTR. For example, recent studies have demonstrated that NF-κB activation by TNF-α results in the nuclear translocation of IKK1, which subsequently promotes phosphorylation of serine 10 on the tail of H3 histones surrounding NF-κB-regulated genes (40, 41). This post-translational modification by IKK1 may help to promote changes in chromatin structure associated with transcriptional activation. Whether IKK1 or other recognized serine 10 kinases like Rsk2 or MSK1 are similarly recruited to the latent HIV-1 LTR following prostratin stimulation is currently under investigation. Additionally, signal-dependent post-translational modification of RelA by phosphorylation and acetylation may also stimulate transcriptional activation of the repressed HIV LTR by enhancing the transcriptional potential of RelA or by impairing the inhibitory action of resynthesized IκBα (42).

Our studies have also shed light on the proximal signaling components involved in the prostratin response. Because prostratin is a nontumorigenic phorbol ester, we focused initial attention on prostratin-mediated activation of various PKC isoforms. When PKC translocation from the cytoplasm to the membrane was evaluated, we observed that conventional, novel, and atypical isoforms were effectively mobilized, indicating a broad response. However, translocation of PKC α, γ, δ, and ζ by prostratin was attenuated relative to that induced by PMA. This differential activation of PKC isoforms may provide a mechanistic basis for the non-tumor-promoting nature of prostratin. The activation of PKCs appears necessary for prostratin induction of the latent HIV LTR, since the generalized PKC inhibitor Gö6983 blocks prostratin induction of GFP expression in J-Lat cells. We additionally found that the treatment of the prostratin-stimulated cultures with an inhibitor that selectively blocks conventional PKC isoforms, Gö6976, did not impair the response, indicating that conventional PKC isoforms are dispensable for this response. Conversely, the addition of Gö6850, which inhibits both conventional and novel PKC isoforms, effectively inhibited the prostratin response. These findings suggest that the novel subfamily of PKC isoforms may play a key role in induction of the latent HIV LTR by prostratin. Whether prostratin signaling is dependent on a single novel PKC isoform or is rather mediated through multiple isoforms remains an open question. In this regard, the addition of inhibitor TER14687, a selective PKC-θ inhibitor, failed to inhibit prostratin antagonism of latency. These findings raise the possibility of functional redundancy in the novel subset of PKCs.

Previous studies of prostratin antagonism of HIV latency have elucidated the ability of prostratin to stimulate p24 Gag production and release from HAART-suppressed human PBMCs or thymocytes present in a SCID-hu Thy/Liv experimental model (15). These systems contain heterogeneous populations of uninfected, actively infected, and latently infected cells, which complicate examination of the overall efficacy of candidate antilatency compounds. The clonal latently infected J-Lat model has the advantage that changes at the single-cell level can be conveniently monitored by flow cytometry. Our studies in fact show that prostratin induces the transcriptional activation of the latent LTR only in a portion of the population. However, a second round of stimulation activates a response in a similar fraction of the previously unresponsive cells. These findings suggest that repeated rounds of stimulation may be required to purge the entire pool of latently infected cells. Differences in the chromatin environment immediately surrounding the HIV integration locus may play an important role in controlling this functional response as well as the basal and induced levels of transcription (43).

HIV postintegration latency in infected patients is probably most stable within the resting memory population of $CD4^+$ T-lymphocytes. To confirm that our observations within the continuously dividing J-Lat model are applicable to primary resting memory T cells, we analyzed NF-κB induction and its activation of a prototypical NF-κB-responsive gene within sorted primary $CD4^+$ $CD45RO^+$ T-lymphocytes. Our observations demonstrate that prostratin is capable of both activating NF-κB and driving NF-κB-dependent gene expression within this biologically relevant population of cells. Additionally, work

by Kulkosky *et al.* (14, 44) has demonstrated that prostratin is capable of inducing outgrowth of latent viruses from the blood of HAART-suppressed patients. Taken together, the proliferating J-Lat model recapitulates many of the responses observed in primary resting memory T cells.

Complete elimination of the latently infected reservoir in infected patients will undoubtedly be a difficult task. Efforts to eliminate this pool of latently infected cells with interleukin-2 and OKT3 antibodies yielded disappointing results (8, 9). For this lofty goal to be reached, more efficient and less toxic antagonists of HIV latency must be identified, and the variegated nature of the response must be successfully dealt with. Although prostratin is promising on some fronts, its cytotoxic properties may limit its utility in human use. The identification of truly effective antagonists of HIV latency could be propelled by a better understanding of the molecular basis of HIV latency. The J-Lat cells used in these studies provide a powerful cellular model for hypothesis testing and biochemical and molecular analysis. However, it is essential that any promising agents ultimately be vetted for their efficacy in primary CD4 T cells latently infected with HIV.

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Chapter 3

NF-κB1 p50 promotes HIV proviral latency through HDAC recruitment and repression of transcriptional initiation

Early studies of HIV-1 transcriptional regulation were performed largely *in vitro*. Proteins capable of binding regulatory elements contained within the 5' LTR were identified through analysis of electrophoretic mobility shift of radioactively labeled naked HIV-1 DNA fragments [1]. Regions of DNA important for HIV-1 transcriptional regulation were defined through deletion mapping of LTR-reporter fusion constructs [2]. Combined approaches allowed the identification of cellular Sp1 and NF-κB proteins and their cognate DNA binding sites within the HIV-1 LTR as key positively acting transcription factors necessary for robust expression of HIV-1 [3-5]. While these approaches were able to identify interactions that may occur with HIV regulatory elements, they lacked the ability to define which interactions *do* occur with integrated HIV-1 proviruses *in vivo*. DNA footpriniting analyses of integrated HIV-1 proviruses took steps towards understanding of interactions *in vivo*, however these approaches remained suggestive rather than definitive [6].

The chromatin immunoprecipitation (ChIP) assay permits analysis of protein-DNA interactions as they exist *in vivo (reviewed in [7])*. Briefly, cells are treated with formaldehyde, a crosslink-inducing agent that covalently links DNA and protein that are immediately juxtaposed. Specific protein-DNA interactions can then be assessed by immunoprecipitating target proteins with specific antibodies, and probing for DNA of interest by PCR. This approach can be used to assess association of transcription factors, such as NF-κB, basal transcription machinery, such as RNA polymerase II, or modified of DNA-organizing proteins, such as acetylated histones, with a region of genomic DNA [8-10]. Most applications of ChIP analyze promoter or enhancer elements involved in the regulation of cellular gene expression. A more recent application has been the

assessment of RNA polymerase II with protein-coding DNA sequences downstream of regulatory elements, thus allowing real-time assessment of transcriptionally active genes [11].

The integrated HIV-1 LTR has been studied by ChIP in various systems [12-15], however these systems lacked the hallmarks of HIV-1 latency, thus their findings are descriptive of general transcription machinery associated with the active HIV-1 promoter. We were interested in characterizing the transcriptional machinery and histone status associated with the latent HIV-1 regulatory elements, and the changes associated with activation of the latent HIV-1 provirus. Additionally, the role of NF-κB as a strong inducer of HIV-1 transcription coupled with the observation of direct RelA binding to the enhancer elements of HIV-1 prompted further investigation into the role of the NF-κB family of transcription factors and their association with the latent provirus.

NF-κB is generally considered a strong activator of transcription, however, the p50-p50 homodimeric species of NF-κB prominent in the nucleus of unstimulated CD4+ T-cells cells has been reported to have transcriptionally repressive properties [16-18]. NF-κB1 p50 lacks the transcriptional activation domain contained in the carboxy-terminus of RelA, but retains DNA-binding activity through the amino-terminal Rel homology domain. Thus, while p50 is capable of binding κB enhancer sites in promoter regulatory elements, it is incapable of recruiting positively-acting transcription cofactors to promote gene expression. Additionally, p50 has been demonstrated to associate with HDAC1, an association that mediates deacetylation of histones proximal to p50-homodimer-bound κB enhancers, establishing a transcriptionally repressive environment

[19]. This negative action of p50 may serve to tighten transcriptional regulation of κB-regulated genes, ensuring that uninduced promoters are not "leaky".

The goal of this project was to explore the association of NF-κB RelA and p50 with the latent and unstimulated promoters, and to the changes in basal transcription factor recruitment and patterns of histone acetylation linked with these associations. We found that the latent HIV-1 κB enhancer is bound by a p50-containing complex that promotes recruitment of HDAC1 and deacetylation of surrounding histones. This deacetylation inhibits recruitment of RNA polymerase II to the latent HIV-1 promoter and prevents initiation of HIV-1 mRNA transcripts. Induction of NF-κB signaling promotes binding of p50/RelA heterodimers to the κB enhancer elements in the latent HIV-1 LTR, removing the repressive effect p50-homodimer recruitment of HDAC1. Elimination of HDAC1 repression by either chemical inhibition or siRNA knockdown of HDAC1 was insufficient to activate HIV-1 transcription, however, likely as a consequence of an absence of positively-acting transcription factors at the HIV-1 promoter.

These findings suggest the use of dual-pronged approaches in next generation immune activating therapies targeting HIV-1 latency. Combined application of NF-κB-inducing and HDAC-inhibiting therapeutic regimens should prove more effective than either therapy alone. Alternatively, exogenously supplied tat might synergize with HDAC-inhibitors to similarly promote transcriptional activation of latent proviruses. It is, however, unlikely that HDAC-inhibitors alone will be successful at purging the latent pool in patients as the lack of positively-acting transcription factors will cause, in many cases, abortion of transcription after initiation. The recent short-term success of valproic

acid as a clinical antagonist in HIV-1 latency might be more pronounced were mild NF-κB agonists such as TNF-α employed [20].

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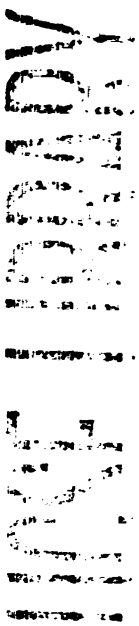

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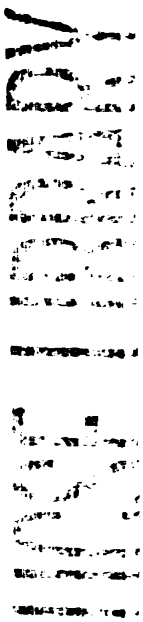

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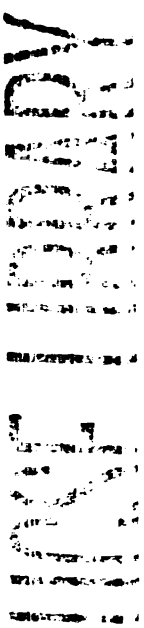

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NF-κB p50 promotes HIV latency through HDAC recruitment and repression of transcriptional initiation

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Cells latently infected with HIV represent a currently insurmountable barrier to viral eradication in infected patients. Using the J-Lat human T-cell model of HIV latency, we have investigated the role of host factor binding to the κB enhancer elements of the HIV long terminal repeat (LTR) in the maintenance of viral latency. We show that NF-κB p50–HDAC1 complexes constitutively bind the latent HIV LTR and induce histone deacetylation and repressive changes in chromatin structure of the HIV LTR, changes that impair recruitment of RNA polymerase II and transcriptional initiation. Knockdown of p50 expression with specific small hairpin RNAs reduces HDAC1 binding to the latent HIV LTR and induces RNA polymerase II recruitment. Similarly, inhibition of histone deacetylase (HDAC) activity with trichostatin A promotes binding of RNA polymerase II to the latent HIV LTR. This bound polymerase complex, however, remains non-processive, generating only short viral transcripts. Synthesis of full-length viral transcripts can be rescued under these conditions by expression of Tat. The combination of HDAC inhibitors and Tat merits consideration as a new strategy for purging latent HIV proviruses from their cellular reservoirs.

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Introduction

A small pool of memory CD4$^+$ T cells latently infected with integrated but transcriptionally silent HIV proviruses has undermined all attempts at curative eradication of virus in HIV-infected patients. Although active replication of HIV can be reduced to undetectable levels by multidrug therapies, the long half-life of these latently infected memory CD4$^+$ T cells predicts that treatment must be continued for more than 60 years for effective clearance of this reservoir (Finzi *et al*, 1997; Siliciano *et al*, 2003). Systemic reseeding of HIV from this latent reservoir appears to play an important role in the recrudescence of viral replication that routinely follows the withdrawal of antiviral therapy in fully suppressed patients. To advance therapeutic strategies beyond an expectation of life-long treatment, novel approaches for dealing with the latent HIV reservoir must be developed (Pomerantz, 2003). An important first step toward this goal is to understand at a molecular level how HIV latency is established and maintained.

HIV latency is associated with a lack of proviral gene expression. Thus, latently infected memory CD4$^+$ T cells differ from their uninfected counterparts only by the presence of the integrated and transcriptionally silent HIV provirus. Expression of the viral genome is regulated by the enhancer and promoter elements contained within the HIV long terminal repeat (LTR) located at the 5′ end of the integrated HIV provirus (see Rohr *et al*, 2003 for review). In the prevailing model of HIV transcription, the RNA polymerase II (RNA Pol II) complex is constitutively bound to the HIV promoter and persistently initiates the synthesis of short RNA transcripts (Kao *et al*, 1987; Ratnasabapathy *et al*, 1990; Yankulov *et al*, 1994). However, in the absence of the virally encoded transactivating protein Tat or cellular activation signals, the processivity of this bound polymerase is sharply limited, leading to the production of abortive short viral transcripts (Laspia *et al*, 1989). Tat binds to an RNA stem–loop structure termed the transactivating responsive element (TAR), which is located at the 5′ end of all initiated viral transcripts (Kao *et al*, 1987), and also recruits cellular cyclin T1 and CDK9 proteins (the p-TEFb complex), which phosphorylate multiple serines in the C-terminal domain (CTD) of RNA Pol II (Zhu *et al*, 1997). These events culminate in an elongating RNA Pol II complex that effectively synthesizes full-length HIV transcripts (Marshall and Price, 1995; Zhou *et al*, 1998). Similarly, induction of transcriptionally active NF-κB strongly promotes recruitment of RelA to κB-binding sites within the LTR, driving the initiation and elongation of HIV transcripts (West *et al*, 2001). Of note, RelA also binds p-TEFb, providing a Tat-independent mechanism for initial RNA Pol II-dependent synthesis of HIV Tat mRNA (Barboric *et al*, 2001).

The prototypical NF-κB transcription factor is a positively acting heterodimeric complex composed of RelA and NF-κB1 p50 (see Hayden and Ghosh, 2004 for review). Both RelA and p50 bind DNA through a shared Rel homology domain; however, RelA also contains C-terminal transcriptional activation domains (TAD), whereas p50 does not. In the absence

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of NF-κB activating stimuli, RelA–p50 heterodimers are bound by a specific inhibitor, IκBα, which prevents this transcription factor from associating with cognate κB enhancers present in NF-κB-responsive target genes. In unstimulated cells, NF-κB1 p50–p50 homodimers are the predominant nuclear species of Rel proteins and bind a range of NF-κB-responsive genes *in vivo*. The ability of NF-κB1 p50 to bind DNA coupled with its lack of a TAD has suggested that p50–p50 homodimers may act as κB-specific transcriptional repressors. Consistent with this notion, p50 homodimers also bind to histone deacetylases (HDACs) and recruit these enzymes to various NF-κB target genes under basal conditions (Zhong *et al*, 2002), where they promote deacetylation of surrounding histones and reduced basal transcriptional activity.

Histones are subject to dynamic covalent modifications that modify gene expression, including acetylation, methylation, ubiquitylation, and ADP-ribosylation (Khorasanizadeh, 2004). Compaction of chromatin associated with HDAC-mediated deacetylation of histone tails can markedly reduce the binding of basal transcription factors to their DNA targets. In contrast, histone acetylation induced by histone acetyltransferases (HATs) is associated with relaxation of chromatin structure, a characteristic of transcriptionally active genes (Kurdistani and Grunstein, 2003). The integrated HIV provirus is assembled into an ordered chromatin structure altered by NF-κB-inducing stimuli or inhibitors of HDAC activity (Pazin *et al*, 1996; Van Lint *et al*, 1996). How this restructuring impacts HIV transcription is unclear.

The exceeding rarity of latently HIV-infected cells coupled with the lack of a distinctive surface marker makes purification and biochemical analysis of these cells impractical. As an experimentally tractable and relevant model of postintegration HIV latency, we have employed Jurkat CD4$^+$ T-cell-based J-Lat clones to explore the molecular underpinnings of HIV latency (Jordan *et al*, 2003). J-Lat cells contain a single, full-length integrated HIV provirus in which GFP has been substituted for *Nef*. This substitution allows rapid assessment of HIV transcriptional activity by cytometric detection of GFP epifluorescence. Our recent studies have shown that NF-κB-inducing agents rapidly promote the binding of RelA to the latent HIV LTR, leading in turn to GFP expression (Williams *et al*, 2004). In the present study, we explored how HIV proviral latency is maintained in J-Lat cells, investigating the potential role of κB-specific transcriptional repressors. Using chromatin immunoprecipitation (ChIP) analyses, we found that the HIV LTR is actively repressed through local histone deacetylation and restriction of promoter access to RNA Pol II mediated by NF-κB1 p50–HDAC1 complex binding.

Results

RNA Pol II binding to the HIV LTR is greatly reduced in latently infected T cells

To examine the potential occupancy of the κB enhancers in the HIV LTR of latently infected human CD4$^+$ T lymphocytes, we first assessed NF-κB1 p50 and RelA binding in ChIP assays using the human J-Lat T-cell model of postintegration HIV latency. J-Lat T cells were incubated with or without TNF-α (20 ng/ml) for 30 min, formaldehyde-crosslinked sheared chromatin extracts were prepared, and these extracts were immunoprecipitated with antibodies specific for RelA, NF-κB1 p50, or RNA Pol II. These immunoprecipitates were then interrogated for the presence of HIV-1 LTR sequences spanning the κB enhancers by PCR (Figure 1B, lanes 1 and 2). More distal HIV sequences (Figure 1B, lanes 3 and 4) and DNA 3 kb upstream (Figure 1B, lanes 5 and 6) or downstream of the β-actin promoter (Figure 1B, lanes 7 and 8) served as specificity controls (Figure 1B, lanes 5 and 6). RelA or RNA Pol II antibodies did not detectibly co-immunoprecipitate HIV LTR DNA in unstimulated samples, suggesting that these factors do not bind to the LTR in unstimulated J-Lat cells *in vivo* (Figure 1B, lane 1). In contrast, unstimulated samples immunoprecipitated with NF-κB1 p50 antibodies were strongly enriched in HIV LTR DNA, suggesting constitutive binding of NF-κB1 p50 to the latent LTR (Figure 1B, lane1). Immunoprecipitation of TNF-α-stimulated samples with anti-RelA and anti-RNA Pol II antibodies led to marked enrichment in HIV LTR DNA, indicating the recruitment of both RelA and RNA Pol II under these stimulated conditions. Binding of NF-κB1 p50 did not appreciably change with TNF-α stimulation (Figure 1B, lane 2).

The absence of detectable binding of RNA Pol II to the latent HIV LTR by ChIP under unstimulated conditions was surprising in view of the prevailing model of a constitutively bound but non-processive polymerase in the absence of Tat or activating stimuli. To confirm that generalized immunoprecipitation of DNA-associated RNA Pol II was not TNF-α dependent in all situations, anti-RNA Pol II immunoprecipitation was performed and followed by interrogation of a constitutively transcribed region of the β-actin gene (β-ACT DS), expected to associate with RNA Pol II under basal conditions. β-ACT DS DNA was readily detected in both untreated and TNF-α-stimulated samples (Figure 1B, lanes 7 and 8, third panel), demonstrating stimulation-independent association of RNA Pol II association with a constitutively transcribed cellular gene. To assess whether RNA Pol II was similarly present on downstream regions of HIV DNA, a real-time indicator of effective polymerase elongation (Sandoval *et al*, 2004), immunoprecipitated samples were assessed for enrichment in HIV *Pol* DNA (DS HIV). RNA Pol II antibodies strongly enriched HIV DS DNA in TNF-α treated but not untreated samples, indicating that cellular activation was required for the appearance of an effective elongating Pol II complex (Figure 1B, lanes 3 and 4, third panel).

The detection of HIV LTR DNA with antibodies specifically reacting with NF-κB1 p50 and RelA after TNF-α induction is consistent with the recruitment of p50 and RelA as a heterodimeric NF-κB complex (p50/RelA). However, this analysis did not exclude the possibility that homodimers of p50 and RelA were recruited to separate HIV LTR DNA fragments. To distinguish between these possibilities, sequential ChIP (seqChIP) with antibodies specific for RelA or p50 was performed. TNF-α stimulated, but not unstimulated, samples incubated with anti-RelA antibodies in both the first and second rounds of immunoprecipitation were highly enriched in HIV LTR DNA (Figure 1C, lane 3). Immunoprecipitation first with anti-RelA and then with anti-p50 showed enrichment in HIV LTR DNA only after TNF-α induction (Figure 1C, lane 4), indicating coassociation of RelA and NF-κB1 p50 with a common fragment of HIV LTR DNA. To confirm this result, we performed seqChIP in the reverse manner, using anti-p50 antibodies in the first immunoprecipitation and

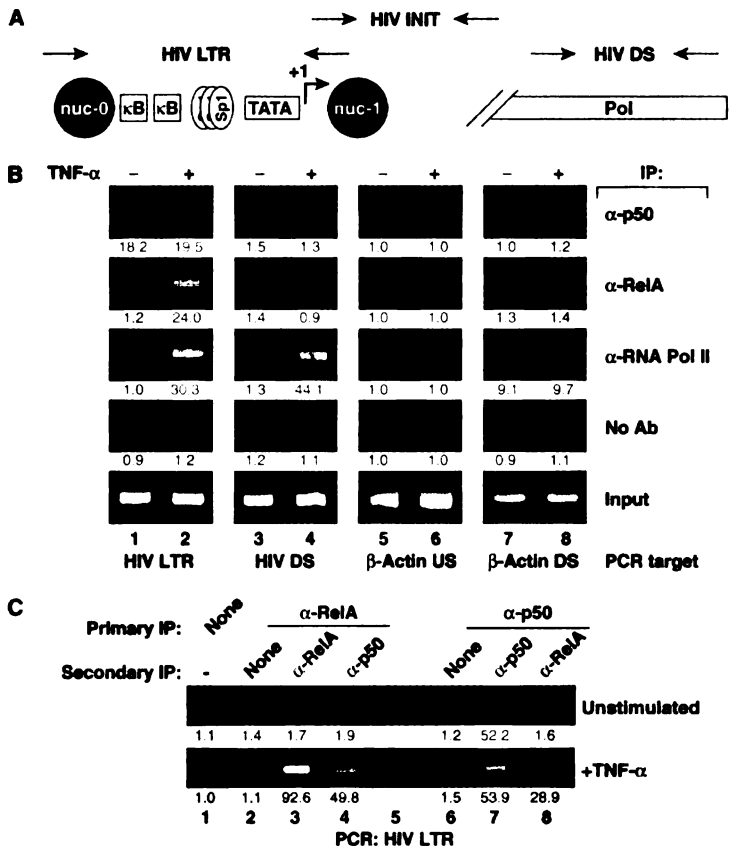


Figure 1 p50/RelA complexes displace NF-κB1 p50 from the latent HIV promoter. **(A)** Schematic of the HIV genome and location of oligonucleotide primers. ChIP primers for analysis of the HIV LTR were designed to span the nuc-0 to nuc-1 region, including the duplicated κB enhancers, the TATA box, and the transcriptional initiation site. Primers for analysis of initiated HIV transcripts were directed against TAR, and primers for downstream analysis were targeted at HIV tat. **(B)** NF-κB1 p50 is constitutively recruited to the HIV-1 promoter, whereas RelA and RNA Pol II are inducibly recruited. Fixed chromatin extracts from J-Lat 6.3 cells treated with 20 ng/ml TNF-α for 30 min or left unstimulated were immunoprecipitated with the indicated antibodies. Samples were assessed for enrichment in HIV LTR DNA, downstream HIV DNA (HIV DS), nonspecific control DNA (β-actin US), or transcriptionally active control DNA (β-actin DS) by UV visualization of PCR products in an ethidium bromide-stained agarose gel. Specific enrichment was quantitated by real-time PCR; mean of three measurements is indicated beneath each band image. Data are representative of three independent experiments. **(C)** A RelA-NF-κB1 p50-containing complex is recruited to the activated HIV LTR. Fixed chromatin extracts from J-Lat 6.3 cells treated as in panel B were immunoprecipitated with the indicated antibodies, and enriched complexes were subjected to a second round of immunoprecipitation with the indicated antibodies. Samples were assessed for enrichment of HIV LTR DNA. Nonspecific enrichment in β-actin DNA was not detected, supporting the specificity of these immunoprecipitations (data not shown). Data are representative of three independent experiments.

anti-p50 or anti-RelA antibodies in the second. In unstimulated cells, HIV LTR binding of p50, but not RelA, was detected; however, in TNF-α-treated samples, RelA-bound HIV LTR DNA was detected in the anti-p50 immunoprecipitates (Figure 1C, lane 8), supporting the conclusion that the HIV LTR in TNF-α-activated cells is bound by p50/RelA heterodimers.

NF-κB1/p50 knockdown induces increased basal transcription of latent HIV

In view of the constitutive binding of NF-κB1 p50 to the latent HIV LTR, we investigated the possibility that p50, which lacks a transactivation domain, might function as a transcriptional repressor of the HIV LTR, thereby reinforcing HIV latency in these cells. To assess this possibility, a small hairpin RNA (shRNA) targeting NF-κB1 p50 was cloned into an expression vector coexpressing mouse MHC class I $H2K^k$, the latter permitting rapid identification of successfully transfected cells. J-Lat cells were transfected with various quantities of p50-shRNA vector, stained for $H2K^k$ expression, and $H2K^k$-expressing cells were enriched by cell sorting. Whole-cell lysates were prepared and analyzed by immunoblotting for NF-κB1 p50 expression. Cells transfected with the p50-targeted shRNA vector exhibited an shRNA dose-dependent decrease in NF-κB1 p50 expression (Figure 2A).

HIV gene expression within these p50-shRNA-transfected cells was examined by flow cytometric analysis of LTR-driven GFP expression in J-Lat cells. In contrast to the induction achieved with TNF-α stimulation, p50-shRNA transfection was not sufficient to drive multi-log increases in GFP

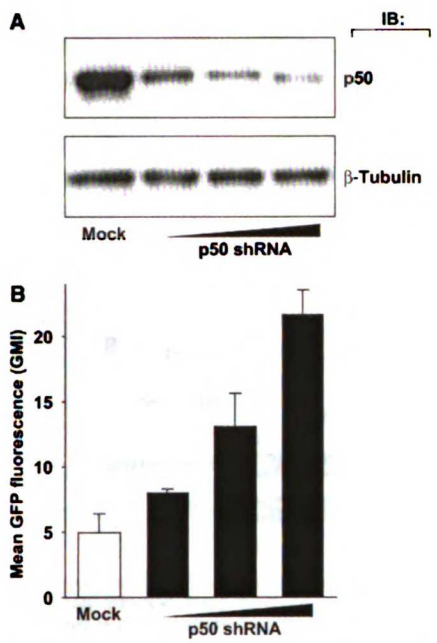


Figure 2 NF-κB1 p50 inhibits basal transcription of HIV in latently infected T cells. (**A**) Transient reduction of NF-κB1 p50 expression with anti-p50 shRNA vector. J-Lat 6.3 cells were cotransfected with an shRNA vector directed against NF-κB1 p50 and a plasmid expressing the cell-surface H-2K^k marker to identify transfected cells. H-2K^k-expressing cells were sorted and lysed 72 h after transfection, and samples were assessed for NF-κB p50 by Western blot. Data are representative of three independent experiments. (**B**) Reduction of NF-κB1 p50 expression is associated with increased basal HIV expression. Cells were treated as in panel A, and H-2K^k-expressing cells were assessed for HIV-LTR-driven expression of GFP by FACS. Experiments were conducted in triplicate; error bars represent standard deviation. Data are representative of three independent experiments.

expression (data not shown); however, cells transfected with p50-shRNA consistently displayed higher GFP fluorescence as measured by increased mean geometric fluorescence (Figure 2B). Induction of p50-shRNA-transfected cells with TNF-α produced a GFP expression response in a similar percentage of the cells as observed with untransfected cells (data not shown). Taken together, these data suggest that a modest increase in HIV transcription occurs following 'knockdown' of p50 expression in the absence of additional cellular stimulation.

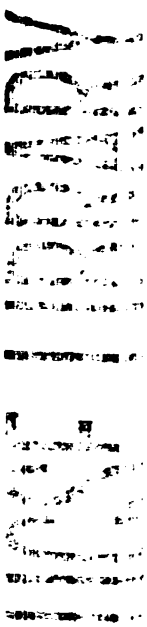
HDAC1 is recruited to the latent HIV promoter and is inducibly removed

NF-κB1 p50 has been suggested to act as a transcriptional repressor not only because it lacks a TAD but also because it assembles with HDAC1. HDAC1 action is associated with repressive changes in chromatin structure, leading to diminished transcription (Zhong *et al*, 2002). To assess whether p50 effectively recruits HDAC1 to the latent HIV LTR, J-Lat chromatin extracts were immunoprecipitated with specific HDAC1 antibodies. The immunoprecipitates were enriched in HIV LTR DNA, indicating that HDAC1 is associated with the latent HIV LTR (Figure 3A). In contrast, immunoprecipitation of HDAC4 or HDAC7 did not yield similar enrichment in HIV LTR DNA (data not shown). To assess the effect of cellular activation and the recruitment of p50/RelA complexes to the HIV LTR on HDAC1 binding, chromatin extracts from TNF-α-treated cells were analyzed by ChIP. Levels of HIV LTR DNA detected with anti-HDAC1 antibodies were markedly decreased in TNF-α-treated samples (Figure 3A). This finding suggests that an HDAC1-containing complex is inducibly depleted from the HIV LTR upon TNF-α induction, consistent with the notion that HDAC1 is recruited to the HIV LTR by a transcriptionally repressive p50–p50 complex constitutively bound to the HIV LTR under basal conditions, which dissociates upon induction of p50/RelA binding. Similar results were observed with PMA stimulation, indicating that NF-κB-inducing stimuli with alternate upstream signaling intermediates correspondingly regulate transcription factor recruitment to the HIV LTR (Supplementary Figure 1).

HDAC1 recruitment to the HIV LTR was strongly reduced by TNF-α treatment, but binding of the deacetylase was not entirely eliminated. To assess whether the residual HDAC1 is bound to the HIV LTR in concert with RelA, seqChIP assays were performed. Untreated chromatin extracts were first immunoprecipitated with anti-HDAC1 antibodies and then with anti-p50 antibodies. The immunoprecipitates were highly enriched in HIV LTR DNA, supporting the notion that NF-κB1/p50 and HDAC1 exist in an interacting complex on the latent HIV promoter (Figure 3B, lane 4, top panel). TNF-α stimulation reduced this enrichment (Figure 3B, lane 4, bottom panel), likely reflecting the replacement of p50 homodimers with p50/RelA heterodimers (Figure 3B, lanes 9 and 11). Secondary immunoprecipitation of anti-HDAC1-immunoprecipitated samples with anti-RelA or anti-RNA Pol II antibodies revealed no detectable enrichment in unstimulated or TNF-α-induced samples, suggesting that HDAC1 is excluded from RelA- or RNA Pol II-containing complexes (Figure 3B, lanes 5 and 6). In contrast, after TNF-α stimulation, sequential immunoprecipitation of p50 and RelA or p50 and RNA Pol II complexes was highly enriched in HIV LTR DNA (Figure 3B, lanes 11 and 12). Thus, the residual HDAC1 present in the TNF-α-treated samples likely reflects either continued binding in the subset of cells that did not respond to the stimulus or recruitment of HDAC1 to the promoter through its association with a different factor.

Inhibition of HDAC activity is associated with RNA Pol II recruitment to the latent HIV LTR and transcriptional initiation but not elongation

To further assess the role of p50-dependent recruitment of HDAC1 in repression of HIV transcription, J-Lat cells were treated with trichostatin A (TSA), a cell-permeable chemical inhibitor of class I/II HDACs. Analysis of GFP expression revealed a fourfold increase in GFP expression as measured by mean geometric fluorescence, a pattern similar to that observed with p50-shRNA (data not shown). One key function of HDACs is the deacetylation of local histone tails, which alters chromatin structure and is associated with impaired transcription of resident genes. To assess the acetylation status of the histones surrounding the latent HIV LTR before and after activation, chromatin extracts were prepared from J-Lat cells treated with TSA or TNF-α and immunoprecipitated with antibodies specific for acetyl-K14 histone H3.



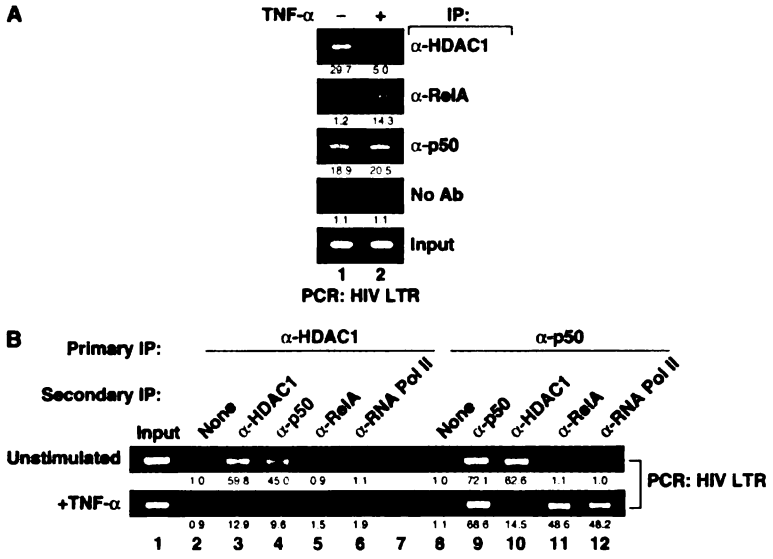


Figure 3 HDAC1 recruitment and loss from the latent HIV LTR RNA Pol II complexes. (**A**) HDAC1 is present on the latent HIV-1 promoter and is lost when T cells are activated. Fixed chromatin extracts from J-Lat 6.3 cells treated with 20 ng/ml TNF-α for 30 min or left untreated were immunoprecipitated with the indicated antibodies. Samples were analyzed for enrichment in HIV LTR DNA by UV visualization of PCR products on an ethidium bromide-stained agarose gel. Specific enrichment was quantitated by real-time PCR; mean of three measurements is indicated beneath each band image. (**B**) HDAC1 is excluded from RelA- or RNA Pol II-containing complexes on the HIV-1 LTR. Fixed chromatin extracts from J-Lat 6.3 cells treated as in panel A were immunoprecipitated with the indicated antibodies and enriched complexes were subjected to a second round of immunoprecipitation with the indicated antibodies. Data are representative of three independent experiments.

Samples treated with TNF-α or TSA, but not untreated controls, were strongly enriched in HIV LTR DNA (Figure 4A, top panel). K14 acetylation of histone H3 is associated with a less compacted chromatin structure, which correlates with increases in the access of basal transcription factors to DNA and transcriptional activation.

To assess the effect of HDAC inhibition on the association of basal transcription factors with latent HIV LTR DNA, extracts were immunoprecipitated with RNA Pol II antibodies. Both TNF-α and TSA treatments were associated with enhanced binding of RNA Pol II to the HIV LTR (Figure 4A, second panel). Next, we investigated whether the CTD of RNA Pol II was phosphorylated, a change that signifies a fully active elongating polymerase complex. While both TSA and TNF-α treatment induced greater binding of RNA Pol II, only TNF-α induced phosphorylation of its CTD (Figure 4A, third panel). ChIP assays further showed that TSA did not interfere with the binding of RelA or NF-κB1 p50 to the HIV promoter (Figure 4A, fourth and fifth panels, and data not shown).

To test independently whether TSA induction was insufficient to induce RNA Pol II processivity, samples immunoprecipitated with anti-RNA Pol II antibodies were probed for enrichment of downstream HIV DNA. TNF-α stimulation induced strong enrichment of DS HIV, but TSA did not (Figure 4B, top panel).

To explore the functional relevance of the association of RNA Pol II with HIV LTR and DS DNA, initiated versus elongated HIV transcripts were measured by quantitative real-time RT–PCR with probe sets targeting HIV TAR in the 5′ 60 bp or the HIV *Tat* exon 1, roughly 5 kb downstream of the HIV LTR. The specificity of primers and the ability to isolate short transcripts were confirmed through analysis of samples treated with TNF-α alone or in combination with DRB, an inhibitor of CDK9-dependent RNA Pol II phosphorylation (data not shown). To assess the effect of TSA on transcription of HIV from the latent promoter, RNA was extracted from J-Lat cells treated with TNF-α, TSA, and untreated controls, and initiated and elongated transcripts were quantified. TSA induced a fourfold increase in initiated HIV transcripts, but failed to enhance elongated transcripts (Figure 4C, gray bars). In contrast, TNF-α increased initiated transcripts eightfold and elongated transcripts from a level below the detection threshold to over 30 copies per cell (Figure 4C, black bars). These observations coupled with the ChIP data highlight a key role for HDAC-mediated inhibition of RNA Pol II association with the latent HIV LTR in the basal state, and consequent reduction of transcriptional initiation. While inhibition of HDAC activity is associated with increased RNA Pol II binding, this polymerase remains unphosphorylated in its CTD and only capable of generating short viral transcripts.

NF-κB1/p50 regulates HDAC1 recruitment to the latent HIV promoter

To determine if recruitment of HDAC1 to the HIV LTR is dependent on NF-κB1 p50 expression, polyclonal populations of J-Lat cells stably expressing p50-shRNA or scrambled control shRNA were isolated. Immunoblotting of the p50-shRNA cells revealed marked reduction of NF-κB1 p50 expression compared with the control or parental cell lines (Figure 5A). Immunoprecipitation of chromatin extracts of these cells with anti-p50 antibodies revealed strong enrich-

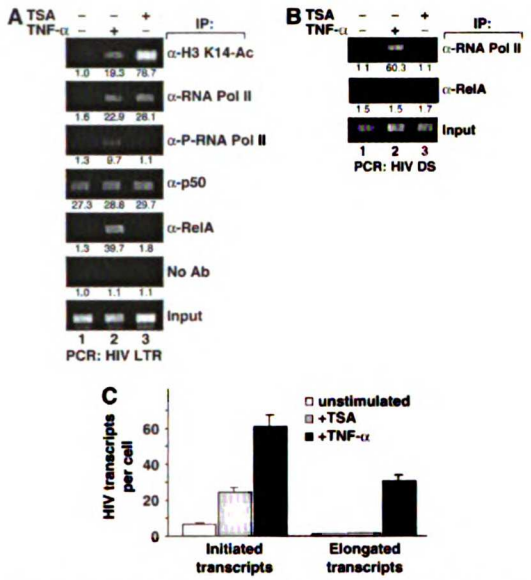


Figure 4 Recruitment of RNA Pol II to the HIV promoter is modulated by histone acetylation. (**A**) Both the activation of NF-κB and addition of the HDAC inhibitor TSA induce histone hyperacetylation and RNA Pol II recruitment to the latent HIV LTR. Fixed chromatin extracts from J-Lat 6.3 cells treated with 20 ng/ml TNF-α for 30 min, TSA for 4 h, or untreated cells were immunoprecipitated with the indicated antibodies. Samples were assessed for enrichment in HIV-LTR DNA by UV visualization of PCR products on an ethidium bromide-stained agarose gel. Specific enrichment was quantitated by real-time PCR; mean of three measurements is indicated beneath each band image. (**B**) TNF-α, but not TSA, induces a processive RNA Pol II complex. Fixed chromatin extracts prepared as in panel A were analyzed for enrichment in downstream HIV DNA (HIV DS). (**C**) TSA induces initiation but not elongation of HIV RNA transcripts. Total RNA was extracted from J-Lat 6.3 cells treated as in panel A, and initiated or elongated HIV RNA transcripts were quantitated by real-time RT–PCR. Bars represent the mean of triplicate samples; error bars represent standard deviation. Data are representative of three independent experiments.

ment in HIV LTR DNA in control cells, but not in p50-shRNA knockdown cells (Figure 5B, top panel). Reduction of NF-κB1 p50 expression did not affect the binding of RelA to the HIV LTR following TNF-α stimulation, nor did it lead to spontaneous RelA binding to the HIV LTR in unstimulated cells (Figure 5B, second panel). In uninduced p50-shRNA cells, the association of HDAC1 with the latent HIV LTR was markedly reduced (Figure 5B, third panel), supporting a key role for p50 in the recruitment of HDAC1 to the latent HIV LTR. Consistent with the predicted effect of the removal of local HDAC activity, anti-acetyl-K14 histone H3 immunoprecipitates revealed marked enrichment in HIV LTR DNA in unstimulated p50-shRNA samples, but not in unstimulated controls (Figure 5B, fourth panel).

The transcriptional consequences of reduced NF-κB1 p50 expression were further explored. Unstimulated p50-shRNA samples immunoprecipitated with anti-RNA Pol II antibodies revealed a strong enrichment in HIV LTR DNA, suggesting increased polymerase binding in the absence of p50. In contrast, no detectable enrichment was observed in the scrambled control shRNA samples. Immunoprecipitation with anti-phospho-RNA Pol II antibodies, however, showed little or no enrichment in HIV LTR DNA in either control- or p50-shRNA-treated samples in the absence of stimulation (Figure 5B, sixth panel).

The observed increase in HIV LTR RNA enrichment in RNA Pol II from cells expressing p50-shRNA and the absence of enrichment in phospho-RNA Pol II suggest that an unphosphorylated, non-elongating RNA Pol II complex is recruited to the latent HIV LTR in the absence of p50. These results were similar to those obtained with TSA-treated cells, suggesting a common mechanism of action. To further test this notion, control- and p50-shRNA stably transfected cells were treated with TSA or left unstimulated, and initiated or elongated HIV transcripts were quantified by real-time PCR. Initiated HIV transcripts were increased by TSA stimulation in control-shRNA cells. In contrast, and like TSA-treated cells, initiated HIV transcripts in untreated p50-shRNA cells were elevated relative to untreated controls (Figure 5C). Further, treatment of p50-shRNA cells with TSA induced only a modest additional increase in the level of initiated transcripts. Either TSA treatment or p50 knockdown was sufficient to promote the accumulation of initiated HIV transcripts but did not appreciably increase elongated transcripts. The fact that reduction of p50 expression was associated with hyperacetylation of histones surrounding the latent HIV promoter is consistent with the observed p50-dependent recruitment of HDAC1 in unstimulated cells. The failure of TSA to further increase initiated transcript formation in p50-shRNA samples adds additional support to the notion that these manipulations alter the same pathway leading to modulation of LTR transcription.

NF-κB1/p50 knockdown or TSA sensitizes latent HIV transcription to Tat

We next assessed whether increased RNA Pol II binding associated with TSA treatment or knockdown of p50 expression confers sensitivity of latently infected cells to the viral Tat transactivator protein. J-Lat cells were transfected with an empty $H2K^k$ expression plasmid DNA or DNA encoding Tat or RelA. Ectopic expression of Tat induced a small subset of latently infected cells to express HIV, as measured by GFP expression. Expression of RelA, however, induced robust HIV gene expression, with ~85% of the transfected cells expressing GFP (Figure 6A). Treatment of transfected cells with a 1 h pulse of TSA strongly enhanced GFP expression in Tat-transfected cell but had little effect on RelA-transfected cells, suggesting that induction of increased initiation of HIV transcripts by TSA sensitizes cells to the transactivating effects of Tat. Similar results were observed in J-Lat clones 8.4 and 15.2 (Supplementary Figure 2), suggesting that a similar mechanism might govern HIV latency in these clones. To examine the influence of TSA treatment on the inherent transactivating potential of Tat on a non-chromatinized substrate, J-Lat cells were transfected with an HIV-LTR luciferase reporter vector in conjunction with control, Tat, or RelA expression vectors, and treated or not with a 1 h pulse of TSA. Both Tat and RelA induced strong upregulation of HIV transcription, with minimal enhancement by TSA (Figure 6B), suggesting that these treatment conditions do not significantly alter the inherent transcriptional activity of Tat and RelA. Taken together, these results indicate that TSA treatment sensitizes latently infected J-Lat cells to Tat action

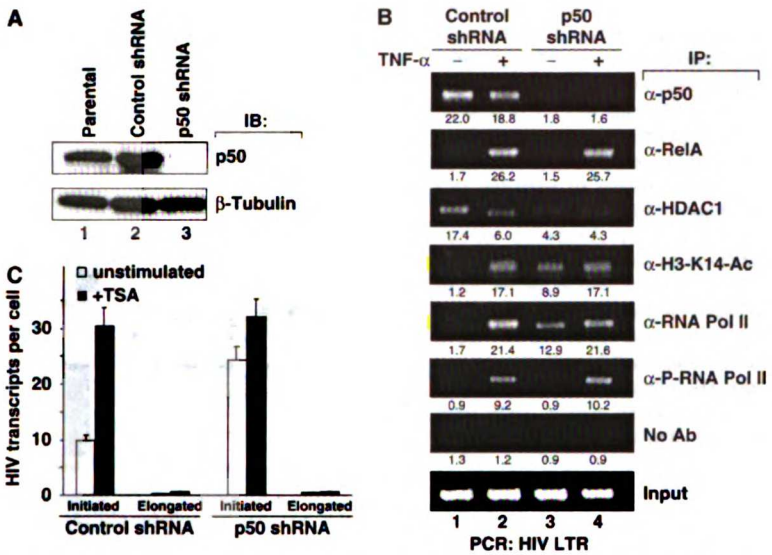


Figure 5 NF-κB1 p50 inhibits basal HIV expression in latently infected T cells by excluding RNA Pol II. (**A**) Stable shRNA knockdown of NF-κB1 p50 in latently infected T cells. Parental J-Lat 6.3 cells or stably transfected clones expressing an shRNA vector directed against a scrambled sequence or NF-κB1 p50 were selected and lysates were analyzed for p50 or control β-tubulin expression. (**B**) Fixed chromatin extracts from J-Lat 6.3 cells stably transfected with scrambled control shRNA or anti-NF-κB1 p50 shRNA treated with 20 ng/ml TNF-α for 30 min or left untreated were immunoprecipitated with the indicated antibodies. Samples were analyzed for enrichment of HIV LTR DNA by UV visualization of PCR products on an ethidium bromide-stained agarose gel. Specific enrichment was quantitated by real-time PCR; mean of three measurements is indicated beneath each band image. (**C**) Cells stably transfected with control- or NF-κB1 p50-shRNA were treated with 100 nM TSA for 2 h or left untreated. Total RNA was extracted, and initiated or elongated HIV RNA transcripts were quantitated by real-time RT–PCR. Bars represent the mean of triplicate samples; error bars represent standard deviation. Data are representative of three independent experiments.

through enhanced recruitment of RNA Pol II, leading to increased transcriptional initiation.

To examine whether NF-κB1 p50 similarly limits the transactivating potential of Tat in latently infected cells, p50- or scramble-shRNA stable cell lines were transfected with control, Tat, or RelA expression vectors. Expression of Tat strongly promoted production of GFP in p50 knockdown cells, but not in the control p50 scramble-shRNA cells (Figure 6C). Further, treatment of Tat-transfected p50-shRNA cells with TSA did not appreciably enhance GFP expression above levels in untreated controls (data not shown), again suggesting that these manipulations likely exert their Tat-sensitizing effects through a common mechanism.

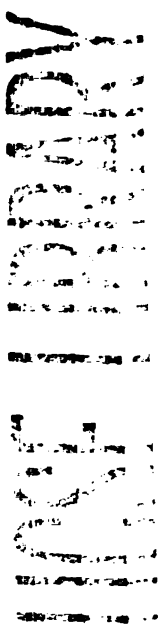
These results strongly favor a model where NF-κB1 p50–HDAC1 complexes promote the maintenance of HIV latency through changes in chromatin structure that impair effective recruitment of RNA Pol II. The absence of RNA Pol II binding to the latent HIV LTR and the consequent failure of transcriptional initiation render these cells unresponsive to Tat.

Discussion

In this study, we used the J-Lat model of postintegration HIV-1 latency to investigate the potential role of κB-specific transcriptional repressors in the maintenance of viral latency. We found that the latent HIV LTR in J-Lat cells is constitutively bound by p50, likely as a p50–p50 homodimer. Additionally, we found that p50 mediates recruitment of HDAC1 to the latent LTR, leading to deacetylation of local histone tails and thus altering the chromatin environment (Figure 7A). The resultant changes in chromatin structure limit the association of RNA Pol II with the latent proviral LTR, emphasizing how p50–HDAC1 repressor complex reinforces transcriptional latency of the integrated HIV provirus. These repressive effects are forfeited either when NF-κB1 p50 expression is knocked down by shRNA or when the enzymatic activity of HDAC1 is inhibited by the addition of TSA (Figure 7C and D). Under these conditions, chromatin structure is altered in a manner that favors increased RNA Pol II binding and enhanced initiation of HIV transcription. Yet, in the absence of transcriptional activators like RelA or the viral Tat protein, the bound polymerase does not effectively elongate, reflecting the absence of cyclin T1–CDK9-mediated phosphorylation of the CTD of the polymerase.

Our findings thus support a model where p50-dependent changes in the chromatin structure of the latent HIV LTR importantly contribute to the maintenance of proviral latency. These results extend and modify a prevailing model of HIV latency, which proposes that constitutive association of RNA Pol II can initiate transcription, but cannot efficiently support elongation in the absence of Tat (Kao *et al*, 1987) or activated NF-κB (West *et al*, 2001). However, as noted earlier, HIV latency is likely multifactorial and blocks could occur at different levels in different latently infected cells (Lassen *et al*, 2004).

The function of p50 as a transcriptional repressor of the latent HIV LTR involving the recruitment of HDAC1 is very



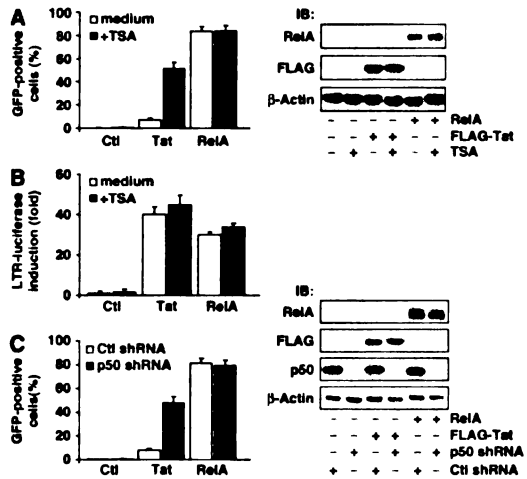


Figure 6 p50 and TSA-sensitive HDACs mediate desensitization of the latent HIV LTR to Tat. (**A**) TSA enhances Tat induction of latent HIV expression. J-Lat cells were transfected with control, Tat, or RelA expression vectors, pulse treated with TSA (400 nM) for 1 h or left untreated, and transfected cells were assessed for GFP expression (left panel). Lysates were prepared and probed for β-actin, RelA, and Tat expression as a control (right panel). Note the low basal sensitivity of J-Lat cells to Tat induction and the sensitization to Tat induced by TSA treatment. (**B**) TSA treatment does not alter inherent Tat transactivating potential. Jurkat cells were transfected with an HIV LTR firefly luciferase reporter vector and a control *Renilla* luciferase vector in conjunction with control, Tat, or RelA expression vectors, pulse treated with TSA (400 nM) for 1 h or left untreated, and relative increase in firefly luciferase activity was quantitated. (**C**) p50 shRNA enhances Tat induction of latent HIV expression. Scramble- or p50-shRNA stable cells were transfected with control, Tat, or RelA expression vectors, pulse treated with TSA (400 nM) for 1 h or left untreated, and transfected cells were assessed for GFP expression (left panel). Lysates were prepared and probed for β-actin, RelA, and Tat expression as a control (right panel). Note the sensitization to Tat expression in p50-shRNA cells and relative lack of additional TSA sensitivity.

similar in many respects to the reported regulation of IL-8 gene expression (Zhong *et al*, 2002), a TSA-responsive host gene (Ashburner *et al*, 2001). The latent HIV and IL-8 promoters are both enriched in acetylated histone H3 after TSA treatment and are constitutively bound to repressive HDAC1/p50-containing complexes, supplanted by active p50/RelA heterodimers (Baek *et al*, 2002). However, the fact TSA is sufficient to drive IL-8 gene expression but is insufficient to activate the latent HIV LTR highlights an important difference in the regulation of these transcription units.

Studies in HeLa cells identified HDAC1 repression of an integrated HIV reporter in HeLa cells through its association with YY1 and LSF (Coull *et al*, 2000; He and Margolis, 2002). In our studies, shRNA knockdown of p50 expression reduced, but did not eliminate, the association of HDAC1 with the HIV LTR. The residual binding of HDAC1 might reflect LSF/YY1-mediated recruitment of the deacetylase to regions downstream of the κB-binding site. Studies of YY1 association with the latent HIV LTR in the J-Lat system did not show a reduction of YY1 binding to the promoter upon activation of gene expression (data not shown). It is possible that both YY1/LSF and p50–p50 homodimers are required to efficiently recruit HDAC1 and that the association of the deacetylase with the LTR involves coordinated recruitment by both factors. Such a scenario could explain the residual HDAC1 binding to the LTR after p50 shRNA knockdown.

The transcriptional activity of NF-κB is itself modulated by dynamic acetylation. For example, RelA acetylation by p300/CBP enhances its nuclear retention and transactivation potential (Chen *et al*, 2001). Additionally, NF-κB1 p50 is inducibly acetylated, promoting increased binding to and transcriptional activation of the COX-2 and iNOS promoters in the context of p50/RelA heterodimers (Deng and Wu, 2003; Deng *et al*, 2003). Of note, we found no change in the binding of NF-κB1 p50 to the latent HIV LTR in TSA-treated samples, nor was binding of RelA induced. In contrast, TSA induced strong acetylation of histone H3, supporting the notion that local HDAC activity promotes the deacetylation of histones arrayed around the HIV LTR. These findings suggest that the enrichment in RNA Pol II at the latent HIV promoter in TSA-treated cells is principally a consequence of histone modification rather than modulation of transcription factor binding.

Our observations are limited to the analysis of various J-Lat T-cell clones containing latent HIV proviruses. Unfortunately, it is not currently possible to identify and purify primary CD4 T cells latently infected with HIV in sufficient numbers to confirm these results in primary cells. However, in a prior study of viral propagation from cultures enriched in latently infected cells, the addition of the HDAC inhibitor valproic acid proved sufficient to rescue low-level viral recovery (Ylisastigui *et al*, 2004). Thus, the processes we have observed in the J-Lat system may reflect important biological events underlying HIV latency *in vivo*. Our observations suggest that HDAC inhibitors might be valuable adjuncts in future strategies aimed at eliminating the latent reservoir in infected patients. Indeed, our findings raise the interesting possibility that HDAC inhibitors in combination with Tat could prove a potent combination for activation of latent HIV proviruses. Such a strategy would be facilitated by the 'protein transducing' properties of Tat, which allow its successful transit across cellular membranes.

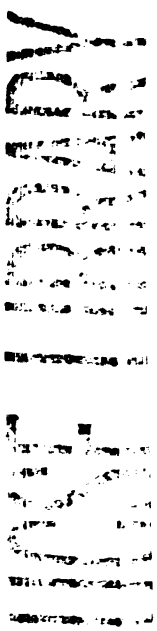
Materials and methods

Cell lines and culture conditions

J-Lat 6.3 cells were maintained in RPMI with 10% fetal calf serum, penicillin, streptomycin, and L-glutamine. For stimulation, cells were treated with 20 ng/ml TNF-α (R&D Systems) or 100 nM TSA (Calbiochem), alone or in combination. For pulsed stimulation with TSA, cells were incubated with 400 nM TSA for 1 h, washed in medium, and suspended in complete medium.

Expression vectors and construction of shRNA vectors

pMACS K^k.II was obtained from Miltenyi Biotech. To 'knock down' p50 expression in J-Lat cells, shRNA vectors were constructed by annealing synthetic DNA oligonucleotide primers with the sequences P50SH_S (5′-GATCCCCGGGGCTATAATCCTGGACTTTCAAGAGAAGTCCAGGATTATAGCCCCTTTTTA-3′) and P50SH_AS (5′-AGCTTAAAAAGGGGCTATAATCCTGGACTTCTCTTGAAAGTCCAGGATTATAGCCCCGGG-3′). A scrambled control version of the same sequence was prepared: SCRAMSH_S (5′-GATCCCCGTCTTACCCTCAGGTCAAATTCAAGAGATTTGACCTGAGGGTAAGACTTTTTA-3′) and SCRAMSH_AS (5′-AGCTTAAAAAGTCTTACCCTCAGGTCAAATCTCTTGAATTTGACCTGAGGGTAAGACGGG-3′). These DNAs were ligated into the *BglII*/*HindIII* sites in digested pTER shRNA cloning vector. The pMACS K^k.II SV40 promoter was eliminated by digestion with *NheI*/*KIe* and then autoligated to produce



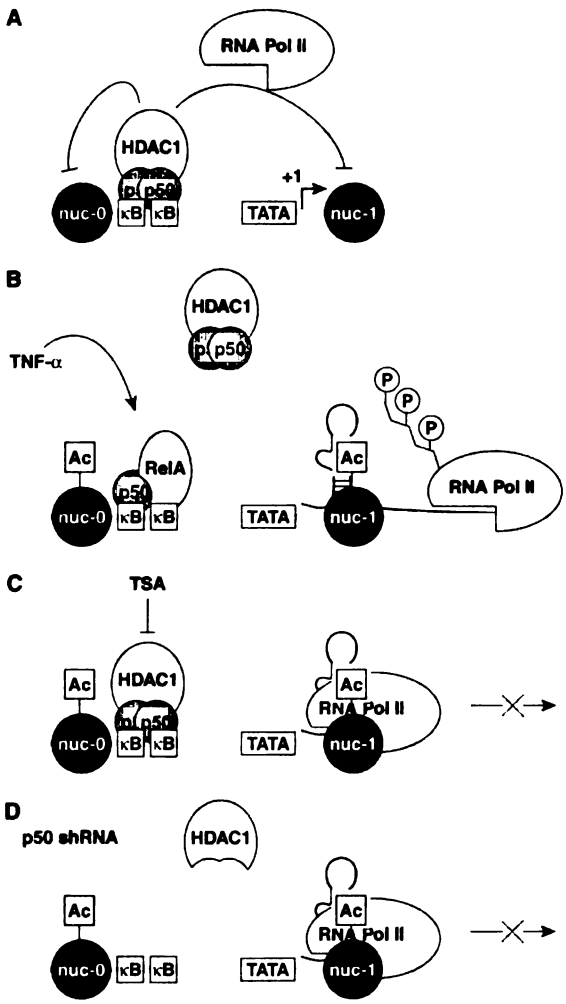


Figure 7 Model of p50-mediated repression of basal HIV expression in latently infected T cells. (**A**) NF-κB1 p50 homodimers bound to the latent HIV-1 promoter recruit HDAC1, which deacetylates regional histones and compacts local histone structure, thereby inhibiting the binding of RNA Pol II. (**B**) TNF-α liberates p50/RelA heterodimers, which displace constitutively bound p50/p50 homodimers present on the HIV LTR, thereby removing HDAC1. The regional shift in favor of HAT activity promotes increased acetylation of surrounding histones, relaxation of chromatin, and increased accessibility to RNA Pol II. Recruitment of CTD kinases by RelA induces transcriptional elongation. (**C**) TSA treatment inhibits HDAC1-mediated deacetylation of regional histones, inducing local histone acetylation, chromatin relaxation, and increased RNA Pol II binding. Under these conditions, RNA Pol II is non-processive owing to the absence of phosphorylation of its CTD. (**D**) shRNA knockdown of p50 displaces the p50–HDAC1 complexes from the latent LTR, promoting local histone acetylation and increased recruitment of RNA Pol II. Similarly, the lack of CTD kinases recruited to the LTR in this context produces a non-processive RNA polymerase complex.

pMACSΔSV40. pTER.NF-κB1/p50 and pTER.Scramble were digested with *Eco*RI/*Hin*dIII, and the resulting fragment containing the modified H1 promoter and shRNA sequence was ligated into pMACSΔSV40 digested with *Eco*RI/*Hin*dIII to produce pMT.NF-κB1/p50 and pMT.Scramble vectors encoding both the targeted shRNA cassette and the H-2K^k marker of transfection driven by the PGK promoter.

Transfection and flow cytometric detection of transfected cells

Transfections were performed by electroporation as described (Williams *et al*, 2004). After 16 h of incubation, the cells were stained with biotin-anti-H-2K^k antibody, washed, and counterstained with streptavidin-APC (Pharmingen). Cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson) using FlowJo software (Treesoft). Cells were sorted with a FACS-DIVA (Becton Dickinson).

To produce stably transfected cell lines, cells were transfected with pMT.NF-κB1/p50 or pMT.Scramble vectors. At 7 days after transfection, H-2K^k-positive cells were enriched by fluorescence-based sorting. This process was repeated four times at 7-day intervals, resulting in >95% of cells expressing the H-2K^k marker of transfection.

Chromatin immunoprecipitation

J-Lat 6.3 T cells were adjusted to 1×10^7 cells/ml and incubated in medium or stimulated with TNF-α (20 ng/ml) for 30 min or TSA (100 nM) for 4 h. ChIP assays were performed as described (Williams *et al*, 2004).

For seqChIP experiments, following an initial round of ChIP and washing, the immunoprecipitated complexes were eluted in buffer containing 1.5% SDS and heated to 65 C for 15 min. Samples were then diluted 10-fold in elution buffer and 10 µl of the second antibody was added. Samples were incubated with agitation at 4 C for 2 h, followed by the addition of salmon sperm DNA/agarose protein A beads at 4 C for 2 h. Antibody–bead complexes were washed and eluted as described in the standard ChIP protocol.

For detection of specific HIV-1 LTR sequences in the ChIP eluates, DNA oligonucleotide primers LTRκBprimer5 (5′-AGGTTTGA CAGCCGCCTA-3′) and LTRκBprimer3 (5′-AGAGACCCAGTACAGG CAAAA-3′) specific for a 203 bp region encompassing the κB binding sites in the HIV LTR were used for PCR amplification. To detect downstream HIV sequences, primers directed against the *Pol* gene with sequences 5HIVDS (5′-TGACTCAGATTGGCTGCAC-3′) and 3HIVDS (5′-AATTTCTACTAATGCTTTA-3′) were employed. To detect sequences in the coding region of the β-actin gene (β-actin DS), oligonucleotide primers with the sequences 5DSBACT (5′-GTCGACAACGGCTCCGGC-3′) and 3DSBACT (5′-GGTGTGGTGCCA GATTTTCT-3′) were used. To detect sequences in the 5′ flanking region of the β-actin gene, primers were targeted at a region 4 kb upstream of the β-actin gene with sequences 5USBACT (5′-GCCAGCTGCAAGCCTTGG-3′) and 3USBACT (5′-GCCACTGG GCCTCCATTC-3′). Amplification was performed using *Taq* polymerase (Qiagen) for 35–40 cycles and products were analyzed on 2.5% agarose gels. Images were acquired with an EagleEye II digital imaging system (Stratagene). Specific enrichment in HIV LTR DNA was quantitated by real-time PCR analysis of ChIP eluates, normalized to enrichment in nonspecific actin DNA. All values are reported in fold-enrichment relative to no-antibody control ChIP eluates.

RNA extraction and analysis of initiated and elongated HIV transcripts

J-Lat 6.3 cells (1×10^6 cells/ml) were treated with TSA (100 nM) or TNF-α (20 ng/ml) for 2 h at 37 C. For analysis of HIV mRNA synthesis in nucleofected primary T cells, RNA was extracted from 0.5×10^6 cells with an RNAWiz kit (Ambion). RNA transcripts were quantitated with the QuantiTect SYBR Green RT–PCR kit (Qiagen). To quantitate viral transcripts, serial dilutions of a quantitated RNA stock of full-length viral genome were used as a reference standard (gift of R Grant). Initiated transcripts were detected with primers HIVTAR5 (5′-GTTAGACCAGATCTGAGCCT-3′) and HIVTAR3 (5′-GTGGGTTCCCTAGTTAGCCA-3′). Elongated transcripts were detected with primers HIVTat5 (5′-ACTCGACAGAGGAGAGCAAG-3′) and HIVtat3 (5′-GAGTCTGACTGTTCTGATGA-3′). β-Actin mRNA copies were quantitated with primers β-actin5 (5′-GTCGA CAACGGCTCCGGC-3′) and β-actin3 (5′-GGTGTGGTGCCAGAT TTTCT-3′) specific for a 239 bp region in the β-actin mRNA and samples were normalized for β-actin copies. Fluorescence profiles were collected on an ABI 7700 real-time thermal cycler and analyzed with SDS v1.91 (Applied Biosystems). The absence of nonspecific bands in RT–PCR products was confirmed on 2% agarose gels.

Immunoblotting analysis

J-Lat 6.3 or stably transfected J-Lat 6.3 cells were pelleted and lysed on ice in egg lysis buffer (ELB) for 20 min and clarified by microcentrifugation. Protein concentration was quantitated using the Bradford protein assay (Bio-Rad), and 10 µg of each sample was added to an equal volume of 2 × Laemmli buffer and heated to 95 C for 5 min. Samples were separated on 10% acrylamide Tris–HCl-buffered SDS–PAGE gels (Bio-Rad), transferred to polyvinylidene difluoride membranes, and immunoblotted with anti-NF-κB p50, RelA (Santa Cruz Biotechnology), anti-FLAG, or anti-β-tubulin (Sigma) antibodies.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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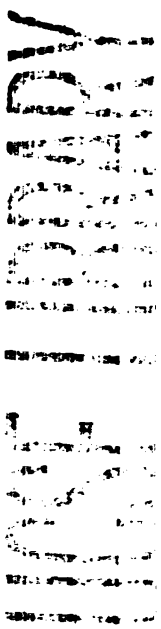

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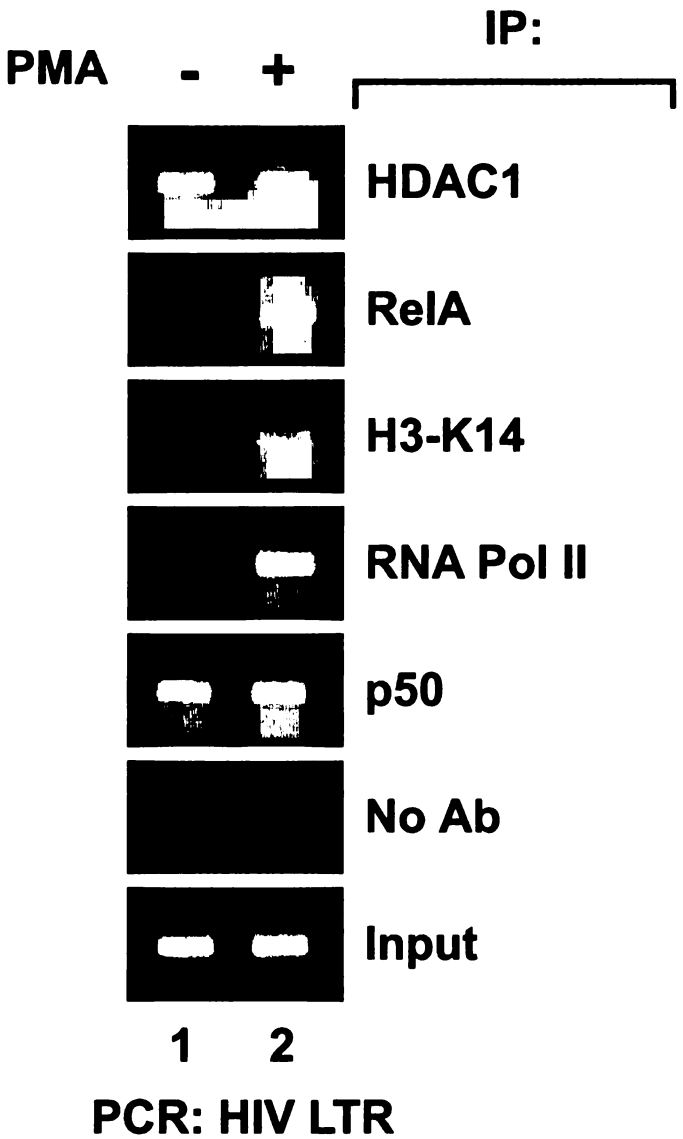
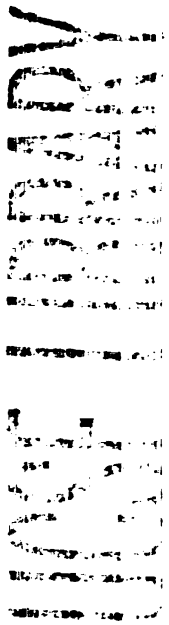
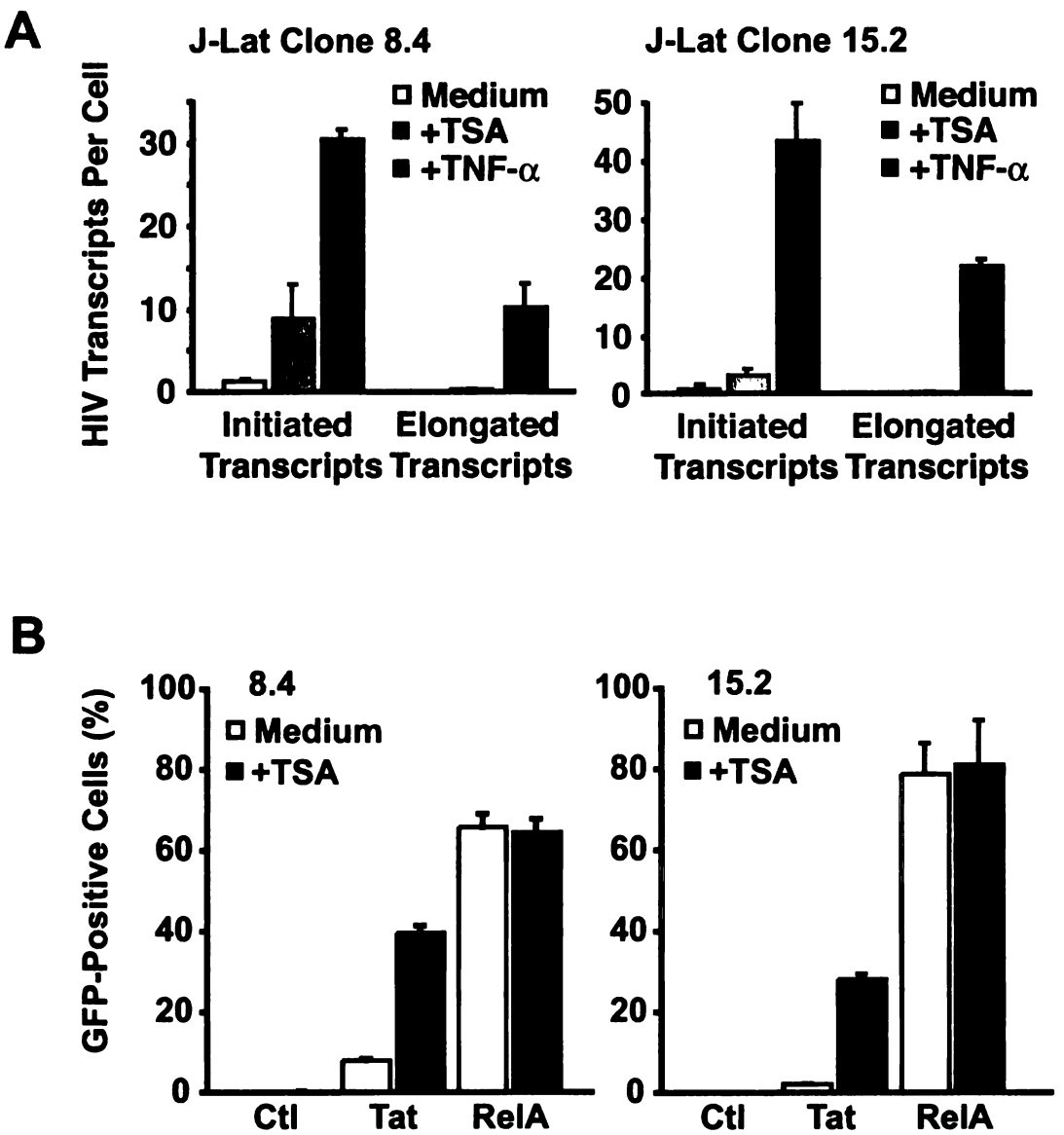


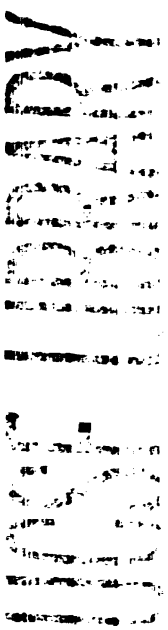


Supplementary Figure 1. PMA induction of NF-κB reduces HDAC1 association with the latent HIV LTR. Fixed chromatin extracts from J-Lat 6.3 cells treated with 20 ng/ml PMA for 30 minutes or left untreated were immunoprecipitated with the indicated antibodies. Samples were analyzed for enrichment in HIV-1 LTR DNA by UV visualization of PCR products on an ethidium bromide stained agarose gel. Patterns of transcription factor association with the HIV LTR induced by PMA are similar to those induced by TNF-α (Figures 3A and 4A).

Williams_Supplementary Figure 2



Supplementary Figure 2. TSA induces increased initiated HIV transcripts in J-Lat clones 8.4 and 15.2 and sensitizes to Tat transactivation of latent HIV. **(A)** Total RNA was extracted from J-Lat 8.4 or 15.4 cells treated with 20 ng/ml TNF-α for 30 min, TSA for 4 hrs, or untreated, and initiated or elongated HIV RNA transcripts were quantitated by real-time RT-PCR. Bars represent the mean of triplicate samples; error bars represent standard deviation. **(B)** TSA enhances Tat induction of latent HIV expression. J-Lat 8.4 (left panel) or 15.4 (right panel) cells were transfected with control-, Tat-, or RelA-expression vectors, pulse treated with TSA (400 nM) for 1 hour or left untreated, and transfected cells assessed for GFP expression 16 hours later. TSA induces initiation but not elongation of HIV RNA transcripts. Bars represent the mean of triplicate samples; error bars represent standard deviation.



Chapter 4

Sustained Induction of NF-κB is Required for Efficient Activation of Latent HIV-1

HIV-1 latency is characterized by the ability of the provirus in an infected cell to remain transcriptionally silent while retaining the potential to become transcriptionally active in response to various cellular cues. Our studies with the J-Lat model of HIV-1 latency demonstrated a key role for NF-κB in the induction of latent HIV-1 gene expression. A broad range of NF-κB agonists including TCR crosslinking by anti-CD3, anti-CD3/CD28, phorbol esters PMA and prostratin, and TNF-α promote renewed transcriptional activity of latent HIV-1 in every J-Lat clone assessed. Our early studies of NF-κB directed activation of latent HIV-1 gene expression focused on the long-term effect of these agonists, analyzing gene expression 16 to 20 hours after induction of NF-κB. This delayed time point was chosen for these analyses because the GFP reporter indicating HIV gene expression requires hours to mature to a fluorescent form, hence real-time analyses of transcriptional activity were not possible with this assay system.

The development of a realtime RT-PCR assay capable of quantitating initiated and elongated transcripts as described in chapter 3 permitted more refined analysis of the kinetics of NF-κB induced transcriptional activation of latent HIV-1. Total RNA can be extracted from cells at any point following stimulation, thus the transcriptional consequences of a stimulus can be assessed minutes after induction rather than days. With this tool established, we set out to understand the kinetics of transcriptional activation of HIV-1 induced by NF-κB.

The events surrounding the transition of HIV-1 latency to a state transcriptional activity are poorly understood. Earlier studies have demonstrated a strong role for NF-κB as an enhancer of HIV transcriptional activity by promoting both transcriptional initiation and elongation (Nabel and Baltimore, 1987; West et al., 2001). The latter

feature is likely mediated by RelA recruitment of P-TEFb, which supports an elongating RNA polymerase II complex in the absence of the otherwise required Tat (Barboric et al., 2001). Existing models hypothesize that RelA is functionally homologous to Tat, and provides the initial elongating cues necessary for a threshold level of Tat to be synthesized, at which point the viral transactivator is capable of directing transcription alone.

Based on these preexisting models, we expected induction of NF-κB to promote rapid and stable accumulation of elongated HIV RNA transcripts, with accelerated accumulation as Tat is synthesized. However, our studies uncovered a surprisingly dynamic relationship of NF-κB with the HIV promoter. Chromatin immunoprecipitation analyses of RelA binding to the HIV-1 LTR reveal multiple waves of binding which are coincident with the recruitment of RNA polymerase II. These waves of RelA binding coincide, in turn, with the general nuclear pool of RelA, which oscillates over time as IκBα levels fluctuate in response to continuous induction of upstream kinases (Covert et al., 2005; Werner et al., 2005). Delayed waves of NF-κB activity are abrogated with transient NF-κB stimulation, a treatment which leads to a single wave of RelA, and surprisingly RNA polymerase II, to the latent HIV-1 LTR. Analyses of HIV LTR-driven GFP expression indicates that transient induction of NF-κB is insufficient to drive efficient expression of HIV, although it is sufficient to drive expression of general κB-responsive genes. To demonstrate this latter point, a κB-DsRed2 reporter was engineered into the J-Lat cell lines, allowing simultaneous assessment of HIV and general κB expression.

Further investigation into the nature of the failure of HIV-1 expression induced by transient induction of NF-κB revealed a relative failure of NF-κB as a promoter of transcriptional elongation. While initiated HIV-1 transcripts rapidly emerge following induction of NF-κB, elongated transcripts do not appreciably emerge until several hours after stimulation, and fail to emerge at all is stimulus is not sustained. The eventual accumulation of elongated transcripts was shown to be dependent on *de novo* synthesis of HIV-1 Tat. Studies seeking to explore the relative deficiency of NF-κB as a promoter of elongation relative to Tat examined the contribution of P-TEFb. Inhibition of P-TEFb ablated the weak accumulation of elongated HIV-1 mRNA transcripts, implying that NF-κB requires P-TEFb for its transcriptional elongating activity. Additionally, analyses of RNA polymerase II phosphorylation at the LTR revealed similar levels of serine-2 phosphorylation induced by NF-κB, and at late time points by Tat, implying that the RNA polymerase serine kinase complex recruited by NF-κB and Tat is similarly capable of inducing this modification. Given the similarities in P-TEFb utilization by RelA and Tat, it is unlikely that differential recruitment or employment of this factor underlies the observed differences in the ability of these transactivators to promote elongation of HIV-1 mRNA transcripts. Additional study will be required to further elucidate the processes employed by Tat which distinguish it from RelA.

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Sustained Induction of NF-κB Is Required for Efficient Activation of Latent HIV-1

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Abstract

Cells harboring infectious, but transcriptionally latent HIV-1 proviruses currently pose an impenetrable barrier for complete eradication of HIV from infected patients. We used the J-Lat experimental model of HIV-1 latency to explore the kinetic relationship of NF-κB induction and the activation of latent HIV-1 proviral gene expression. Chromatin immunoprecipitation analyses revealed an oscillatory and synchronous pattern of RelA and RNA polymerase II recruitment to the HIV-1 LTR following activation of NF-κB by continuous exposure of cells to TNF-α. Transient induction of NF-κB, produced by exposure of cells to TNF-α for 15 minutes, restricted this pattern to single transient phase of RNA polymerase II and RelA recruitment to the LTR. Using these transient stimulation conditions, activation of latent proviral gene expression was markedly impaired. Similarly, the analysis of HIV-1 transcript formation revealed that efficient elongation of HIV-1 transcripts required sustained induction of NF-κB and depends on *de novo* synthesis of Tat. Low-level NF-κB-stimulated viral transcript elongation was blocked by the addition of 5,6-dichloro-1-beta-D-ribobenzimidazole (DRB) suggesting the potential involvement of PTEF-b in this response. Further, serine-2 phosphorylation of RNA polymerase II was detected immediately following NF-κB induction. These results indicate that transient recruitment of P-TEFb to the proximal HIV promoter by NF-κB is not sufficient for efficient elongation of HIV mRNA transcripts and activation of proviral gene expression. Rather, sustained NF-κB activation is essential, likely to promote effective synthesis of Tat.

Introduction

Efforts aimed at eradicating HIV-1 in infected patients with antiviral drug combinations have been thwarted in part by the presence of a reservoir of latently infected T-cells. Latent HIV-1 infection involves successful integration of the provirus into host DNA but a failure of these integrated proviruses to synthesize long viral transcripts (see (Blankson et al., 2002) for review). As a consequence of the failure of viral gene expression, latently infected cells are insensitive to existing antiretroviral therapies yet following their withdrawal, retain the potential to reseed systemic infection when their cellular hosts are activated. The minimal half-life of one latent pool of HIV-1 present within memory CD4 T-cells is 45 months, and assuming an initial pool of 10^5 infected cells, would require over 60 years to eradicate with existing therapies (Finzi et al., 1999; Siliciano et al., 2003). If curative therapies for HIV-1infection are to become a reality, this pool of persistently infected and drug-insensitive virus must be addressed and eliminated (Pomerantz, 2003). A recent clinical study examining valproic acid, a weak histone deacetylase inhibitor, in HIV-1 infected patients demonstrated moderate reductions in the pool of latently infected memory T-cells in selected patients (Lehrman et al., 2005). While a step forward, the reductions achieved were clearly insufficient in magnitude and the response proved transient. New approaches that produce a much larger and durable response are required. Successful identification of new active agents will be facilitated by a more complete understanding of the molecular events that regulate HIV-1 latency.

The principal feature of HIV-1 latency is a failure of viral gene expression, chiefly as a consequence of aborted transcription. Expression of the viral genome is

regulated by the promoter and enhancer elements contained within the long terminal repeat (LTR) located at the 5-prime end of the integrated provirus (see (Rohr et al., 2003) for review). Elongation of HIV-1 mRNA transcripts is strongly dependent on the viral transactivating protein, Tat, which binds to a highly structured region of viral RNAs termed TAR at the 5-prime end of initiated transcripts (Kao et al., 1987). Tat recruits the cellular P-TEFb kinase complex, comprised of cyclinT1 and CDK9 (Zhu et al., 1997), to the HIV-1 promoter and induces serine-2 phosphorylation of C-terminal domain of RNA polymerase II (Parada and Roeder, 1996). This modification promotes efficient transcriptional elongation. Additionally, Tat partners with histone acetyltransferases p300 and P/CAF (Benkirane et al., 1998; Marzio et al., 1998), although the role of these proteins in Tat-induced transcriptional elongation of HIV-1 mRNA transcripts is less clear. Mutation of Tat or TAR underlies the HIV-1 latency observed in U1 and ACH-2 cell lines (Emiliani et al., 1998; Emiliani et al., 1996). Remarkably, infectious virus can be induced from both of these cell lines by a range of NF-κB-inducing agents, indicating that this cellular transcription factor can mimic some of the functions of Tat.

The prototypical NF-κB heterodimer, comprised of RelA and p50, binds to two κB enhancer sites in the HIV-1 LTR positioned immediately upstream of the transcription initiation site (Bohnlein et al., 1988; Perkins et al., 1993; Williams et al., 2005). Induction of NF-κB is associated with both enhanced transcriptional initiation and elongation of HIV-1 mRNA transcripts (Nabel and Baltimore, 1987; West et al., 2001). RelA contains a transcriptional activation domain (TAD), which mediates recruitment of a number of positively-acting transcription factors, including p300 and P/CAF (Sheppard et al., 1999) and the P-TEFb serine kinase complex (Barboric et al., 2001). Recently, we

have demonstrated that RelA-driven displacement of repressive p50-HDAC1 complexes resident on the latent HIV-1 LTR contributes to transcriptional initiation (Williams et al., 2005). RelA-driven recruitment of P-TEFb is thought to underlie initial elongation of HIV-1 mRNA transcripts, permitting synthesis of Tat (Barboric et al., 2001). Additionally, RelA-mediated recruitment of p300 promotes enhanced localized histone acetylation (Ito et al., 2000) and acetylation of Tat itself at lysine-50 (Kiernan et al., 1999).

In the absence of appropriate activating stimuli, NF-κB is predominantly localized within the cytoplasmic compartment and prevented from binding DNA by its association with inhibitory IκB proteins (Davis et al., 1991; Ganchi et al., 1992; Henkel et al., 1992; Zabel et al., 1993). Various stimuli trigger intracellular signaling cascades that coalesce in the activation of the IKK signalsome complex. Activated IKK-mediates phosphorylation of IκBα which in turn promotes polyubiquitylation and proteasome-mediated degradation of this inhibitor (DiDonato et al., 1997; Mercurio et al., 1997; Sun et al., 1993; Zandi et al., 1997). Liberated NF-κB complexes rapidly translocate into the nucleus, bind cognate DNA enhancer loci, and induce gene expression. Because the IκBα promoter contains κB enhancers, NF-κB activation stimulates *de novo* synthesis of the inhibitor. This autoregulatory feedback loop actively limits the duration of NF-κB activation (Sun et al., 1993).

The self-limiting duration of NF-κB activation and the apparent functional homology of RelA and Tat as recruiters of P-TEFb prompted us to further investigate the role of NF-κB in the activation of latent HIV-1 proviruses. The lack of specific identifying markers on latently infected memory $CD4^+$ T-cells in HIV-1-infected patients

precludes use of primary cells for biochemical and molecular studies. As a surrogate experimental model of HIV latency, we have utilized J-Lat cells (Jordan et al., 2003). These cells consist of Jurkat T-cell clones infected with single-copy latent full-length HIV-1 proviruses that express GFP in lieu of Nef. Prior studies using this system identified a histone-acetylation-dependent exclusion of RNA polymerase (Pol) II from the latent promoter and consequent failure in transcriptional initiation as a primary feature underlying HIV-1 latency (Williams et al., 2005).

In the current study, we have explored the interplay between NF-κB and Tat in the activation of latent HIV-1 proviral gene expression. We show that NF-κB and Tat drive transcriptional elongation in a P-TEFb-dependent manner. However, there is striking disparity in the efficiency of elongation promoted by these stimuli. Tat is highly efficient while NF-κB is not. In fact, we find that transient induction of NF-κB is insufficient to activate the latent provirus although other NF-κB-dependent transcription units are efficiently activated. These striking differences argue that additional factors distinct from PTEF-b are utilized by Tat to promote efficient activation of HIV and that sustained NF-κB activation is likely required to ensure effective production of Tat.

Materials and Methods

Cell lines and culture conditions

J-Lat 6.3 cells were maintained in RPMI with 10% fetal calf serum, penicillin, streptomycin, and L-glutamine. For stimulation, cells were treated with 20 ng/ml TNF-α (R&D Systems) or 100 nM trichostatin A (TSA, Calbiochem) alone or in combination. For experiments involving inhibition of PTEF-b, 5,6-dichloro-1-ß-D-

ribofuranosylbenzimidazole, or DRB, (Sigma) was used at 10 ng/ml, and cycloheximide (Sigma) was used at 10 μg/ml. To produce NF-κB-DsRed2-reporter cells, a lentiviral vector was constructed containing 4 tandem consensus κB binding sites positioned upstream of the coding sequence for DsRed2. This construct was cotransfected with VSV-g envelope into HEK 293T cells, and virus-containing supernatants were harvested and concentrated with Centricon filtration units (Millipore). Parental Jurkat or J-Lat 6.3 cells were infected with concentrated viral stocks and incubated 72 hours, and DsRed2-negative cells were sorted for by FACS. After negative sorting, cells were treated with TNF-α and incubated for 24 hours, and cells with TNF-α-inducible DsRed2 expression were selected for by flow cytometry, Jurkat-κB-Red, and J-Lat-6.3-κB-Red.

Chromatin Immunoprecipitation

J-Lat 6.3 T cells were adjusted to 1 x 10^7 cells/ml and incubated in medium or stimulated with TNF-α (20 ng/ml) for 30 minutes or TSA (100 nM) for 4 hours. ChIP assays were performed as described (Williams et al., 2004). Antibodies employed were RelA (sc-109), RNA polymerase II (sc- sc-899), ser-2-RNA polymerase II (H5))

For detection of specific HIV-1 LTR sequences in the ChIP eluates, DNA oligonucleotide primers LTRκBprimer5 (5'-AGGTTTGACAGCCGCCTA-3') and LTRκBprimer3 (5'-AGAGACCCAGTACAGGCAAAA-3') specific for a 203-bp region encompassing the κB binding sites in the HIV-1LTR were used for PCR amplification. To detect downstream HIV-1sequences, primers directed against the *pol* gene with sequences 5HIVDS (5'-TGACTCAGATTGGCTGCAC-3') and 3HIVDS (5'-

AATTTCTACTAATGCTTTA-3') were employed. To detect sequences in the coding region of the β-actin gene (β-actin DS), oligonucleotide primers with the sequences 5DSBACT (5'-GTCGACAACGGCTCCGGC-3') and 3DSBACT (5'-GGTGTGGTGCCAGATTTTCT-3') were used. To detect sequences in the 5' flanking region of the β-actin gene, primers were targeted at a region 4 kb upstream of the β-actin gene with sequences 5USBACT (5'-GCCAGCTGCAAGCCTTGG-3') and 3USBACT (5'-GCCACTGGGCCTCCATTC-3'). Amplification was performed using *Taq* polymerase (Qiagen) for 35-40 cycles and products were analyzed on 2.5% agarose gels. Images were acquired using an EagleEye II digital imaging system (Stratagene).

RNA extraction and analysis of initiated and elongated HIV-1transcripts

J-Lat 6.3 cells (1 x 10^6 cells/ml) were treated with TSA (100 nM) or TNF-α (20 ng/mL) for 2 hours at 37°C. For analysis of HIV-1mRNA synthesis in nucleofected primary T cells, RNA was extracted from 0.5x10^6 cells with an RNAWiz kit (Ambion). RNA transcripts were quantitated using the QuantiTect SYBR Green RT-PCR kit (Qiagen). To quantitate viral transcripts, serial dilutions of a quantitated RNA stock of full-length viral genome were employed as a reference standard (gift of R. Grant). Initiated transcripts were detected with primers HIVTAR5 (5'-GTTAGACCAGATCTGAGCCT-3') and HIV-1TAR3 (5'-GTGGGTTCCCTAGTTAGCCA-3'). Elongated transcripts were detected with primers HIVTat5 (5'-ACTCGACAGAGGAGAGCAAG-3') and HIVtat3 (5'-GAGTCTGACTGTTCTGATGA-3'). β-actin mRNA copies were quantitated with primers β-actin5 (5'-GTCGACAACGGCTCCGGC-3') and β-actin3 (5'-

GGTGTGGTGCCAGATTTTCT-3') specific for a 239-bp region in the β-actin mRNA and samples were normalized for β-actin copies. Fluorescence profiles were collected on an ABI 7700 real-time thermocycler and analyzed with SDS v1.91 (Applied Biosystems). Absence of non-specific bands in RT-PCR products was confirmed on 2% agarose gels.

Immunoblotting analysis

J-Lat 6.3 or stably transfected J-Lat 6.3 cells were pelleted and lysed on ice in ELB for 20 min and clarified by microcentrifugation. Protein concentration was quantitated using the Bradford protein assay (BioRad), and 10 μg of each sample was added to an equal volume of 2x Laemmli buffer and heated to 95°C for 5 min. Samples were separated on 10% acrylamide Tris-HCl buffered SDS-PAGE gels (BioRad), transferred to polyvinylidene difluoride membranes, and immunoblotted with anti-NF-κB p50, RelA (Santa Cruz Biotechnology), anti-FLAG or anti-β-tubulin (Sigma) antibodies.

Electrophoretic Mobility Shift Assay

J-Lat 6.3 cells were adjusted to 2 x 10^6 cells/ml and stimulated with 10 ng/ml TNF-α for various times. After stimulation, nuclear extracts were prepared as described (20). For analysis of κB enhancer binding proteins, 2.5 μl of the nuclear extracts were incubated with poly(dI-dC) and salmon sperm DNA for 10 min, followed by the addition of [γ-^{32}P]ATP-labeled consensus κB or Oct-1 enhancer oligonucleotides (Promega). Samples were separated on nondenaturing gels and analyzed by autoradiography.

Transfection and Flow Cytometric Detection of Transfected Cells

Transfections were performed by electroporation as described (Williams et al., 2004). After 16 hours of incubation, cells were stained with biotin-anti-H-$2K^k$ antibody, washed, and counter-stained with streptavidin-APC (Pharmingen). Cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson) with FlowJo software (Treesoft). Cell sorting was performed using a FACS-DIVA (Becton Dickinson).

Results

Continuous stimulation of NF-κB activation promotes oscillating and synchronous binding of RelA and RNA polymerase II with the latent HIV-1 LTR

Prior studies of the latent HIV-1 LTR revealed TNF-α-inducible recruitment of RNA Pol II (Williams et al., 2005). To further characterize the events underlying activation of the latent HIV-1 LTR, we sought to define the kinetics of RNA Pol II recruitment after activation of latent HIV-1 proviruses. Chromatin immunoprecipitation (ChIP) assays with the human J-Lat T cell model of postintegration HIV-1 latency were used to study interaction of RNA Pol II with the HIV-1 LTR *in vivo*. Sheared formaldehyde-crosslinked chromatin extracts prepared from J-Lat cells left untreated or stimulated with 20 ng/ml TNF-α for various times were immunoprecipitated with antibodies specific for RNA Pol II. The abundance of HIV-1 LTR DNA in immunoprecipitates was assessed by ethidium bromide visualization of PCR products amplified with primers specific for the HIV-1 LTR. In extracts from untreated cells, RNA Pol II immunoprecipitates failed to specifically enrich HIV-1 LTR DNA, indicating a lack of binding of this polymerase complex to the unstimulated latent HIV-1 LTR (Figure 1A). In contrast, RNA Pol II

immunoprecipitates from extracts of TNF-α-stimulated cells were enriched in HIV-1 LTR DNA after 15 min of stimulation. This enrichment increased at 30 min after TNF-α stimulation. Strikingly, a marked reduction in enrichment was consistently noted at 1-hour after TNF-α stimulation. Increased enrichment in HIV-1 LTR DNA in RNA Pol II immunoprecipitates was again observed in extracts of cells stimulated with TNF-α for 2, 4, and 6 h. Importantly, the levels of input HIV-1 LTR DNA were similar at each time (Figure 1A).

The reduction in RNA Pol II association with the HIV-1 LTR after 1 h of TNF-α stimulation was unexpected and suggested a dynamic association and dissociation of this enzyme and perhaps other cofactor(s) necessary for effective recruitment of RNA Pol II. In this regard, RelA has been implicated as one such cofactor. Accordingly, we assessed the pattern of RelA binding to latent HIV-1 LTR over time after TNF-α stimulation. RelA immunoprecipitates from extracts of untreated cells displayed no specific enrichment in HIV-1 LTR DNA. Extracts of TNF-α-stimulated cells were specifically enriched in HIV-1 LTR DNA in a bimodal pattern, with apparent synchrony to the observed pattern of RNA Pol II association (Figure 1A). Peaks in specific enrichment in HIV-1 LTR DNA were observed in RelA immunoprecipitates of extracts of cells treated for 30 min and 4 h, and a nadir in enrichment was apparent one hour after TNF-α stimulation.

The dynamic pattern of RelA association and dissociation with the latent HIV-1 LTR after TNF-α stimulation resembled oscillatory patterns of NF-κB recruitment recently reported on various host promoters occurring in response to various NF-κB-inducing

stimuli (Covert et al., 2005; Nelson et al., 2004; Werner et al., 2005). This oscillation is attributed to κB-dependent induction of IκBα gene expression, which inhibits DNA binding and promotes nuclear export of RelA. Continued stimulation of the upstream IκB kinases (IKKs) induces subsequent rounds of IκBα phosphorylation and degradation, leading to delayed oscillating waves of nuclear accumulation of NF-κB. To examine whether this process might underlie the pattern of RelA recruitment to the latent HIV-1 LTR, the abundance of IκBα and nuclear RelA after TNF-α stimulation were assessed by immunoblot analysis. Following TNF-α application, expression of IκBα was rapidly reduced, however resynthesis of the inhibitory protein was apparent after 1 h. The level of IκBα expression after 2 hours of TNF-α treatment appeared to be slightly reduced relative to untreated samples (Figure 1B). Nuclear abundance of RelA was inversely correlated with IκBα levels, with a peak in nuclear RelA at 30 min of TNF-α stimulation, followed by a nadir at 1 hour, and a renewed peak at 4 h of stimulation (Figure 1C). The overall pattern of kinetics bore great similarity to those observed in the ChIP analyses, supporting the argument that the oscillating nuclear abundance of RelA underlies the pattern observed in ChIP analyses.

Transient induction of NF-κB induces synchronous unimodal association of RelA and RNA polymerase II with the latent HIV-1 LTR

We next investigated the contribution of the first wave of RelA recruitment to expression of latent HIV-1 without subsequent delayed waves. Because the delayed oscillatory nuclear recruitment of RelA depends upon continued induction of NF-κB signaling, we reasoned that a transient stimulation of the cells with TNF-α would induce

a single cycle of RelA activity. To test this notion, J-Lat 6.3 cells were stimulated with TNF-α for 15 min, washed extensively, and returned to culture for various times. The pulsed administration of TNF-α induced rapid degradation of the inhibitor, with overall levels falling to levels similar to those observed with continuous TNF-α stimulation (Figure 2A). Resynthesis of IκBα was apparent 1 h after TNF-α pulse treatment, and in contrast to continuous stimulation, expression levels thereafter appeared to remain slightly higher than those observed in lysates of untreated cells. Correspondingly, early nuclear accumulation of RelA was observed in TNF-α-pulsed samples with kinetics similar to samples treated continuously with TNF-α, and nuclear RelA was not apparent 1 hour after the TNF-α pulse (Figure 2B). Taken together, these results indicate that delayed waves of nuclear RelA are not induced by transient stimulation of the cells for 15 minutes with TNF-α.

To examine the impact of transient induction of NF-κB on transcriptional regulation of latent HIV-1, sheared chromatin extracts from TNF-α pulsed samples were immunoprecipitated with antibodies specifically recognizing RelA or RNA Pol II, and enrichment in HIV-1 LTR DNA was assessed. Consistent with the predicted transient induction of NF-κB, RelA immunoprecipitates were enriched in HIV-1 LTR DNA in a unimodal pattern, with a peak in enrichment observed at 1 hour after the TNF-α pulse (Figure 2C). RNA Pol II immunoprecipitates also displayed a similar unimodal pattern of enrichment in HIV-1 LTR DNA.

Transient induction of NF-κB is insufficient to induce robust expression of latent HIV-1

The transient nature of RNA Pol II immunoprecipitate enrichment in HIV-1 LTR DNA after pulsed TNF-α stimulation prompted assessment of a potential decline in HIV gene expression. To study this possibility, J-Lat 6.3 cells were pulsed or treated continuously with TNF-α and cultured for 16 h, and HIV-1-transcription dependent expression of GFP was assessed by flow cytometry. Expression of GFP in unstimulated J-Lat cells was minor while continuous stimulation with TNF-α induced robust GFP expression in 36% of cells (Figure 3A). In contrast, pulsed TNF-α stimulation induced only 3.8% of cells to express GFP. Similar results were observed in J-Lat clones 8.4 and 15.2, indicating that this effect was observed in multiple J-Lat clones (data not shown). As a whole, these observations suggest that sustained induction of NF-κB is necessary for the activation of robust latent HIV-1 gene expression.

The relative lack of latent HIV-1 gene expression in response to transient TNF-α stimulation of NF-κB could reflect a unique property of the HIV-1 promoter, or could reflect a more general property of many κB-responsive genes. To further explore this quaestion, Jurkat T cells were transduced with a lentiviral reporter vector expressing DsRed2 regulated by a tandem array of κB sites, producing a stable population of κB reporter cells termed JκRed. Without stimulation, DsRed2 expression in JκRed cells was very low, apparently reflecting minimal basal activity of this reporter, (Figure 3B). After16 h of continuous TNF-α stimulation, 98% of the JκRed cells expressed DsRed2, indicating induction of κB-specific gene expression in virtually the entire cell population. Similarly, and in striking contrast to expression of HIV-1-driven GFP, 95% of JκRed cells stimulated with TNF-α for 15 minutes exhibited DsRed2 expression 16 h later.

To exclude the possibility of confounding mutations and to confirm that similar transient inducibility of κB-dependent gene expression is possible in J-Lat cells, stable populations of J-Lat 6.3 cells containing κB-DsRed2 reporter were prepared, pulsed or continuously stimulated with TNF-α, and analyzed after 16 h of culture by flow cytometry. Like JκRed cells, J6.3κRed cells exhibited a modest background of DsRed2-expressing cells (Figure 3C). 97% of cells continuously stimulated with TNF-α expressed κB-driven DsRed2, and 30% expressed HIV-1LTR-driven GFP. Similarly, 93% of TNF-α-pulsed J6.3κRed cells exhibited DsRed2 expression, however only 1.7% of these cells expressed GFP. These results indicate that the failure of robust expression of HIV-1in response to transient NF-κB induction is not a general feature of κB-dependent transcription, but likely reflects properties unique to the latent HIV-1 LTR.

TNF-α induction of efficient transcriptional elongation of latent HIV-1 is delayed and requires sustained NF-κB activation

We next examined the abundance of initiated and elongated HIV-1 mRNA transcripts in J-Lat cells pulse-stimulated or continuously activated with TNF-α at various time points by quantitative real-time RT-PCR. Continuous TNF-α treatment induced a rapid 10-fold increase in initiated HIV-1 mRNA transcripts, plateauing temporarily at 1 h, and increasing exponentially thereafter (Figure 4A). In contrast, elongated HIV-1 mRNA transcripts were only modestly induced during the first four hours of TNF-α treatment, with exponential increases becoming apparent at later time points.

Transient induction of NF-κB induced formation of initiated HIV-1 mRNA transcripts with kinetics similar to that observed at early time points in continuously

stimulated cells. However, after 1 h, initiated transcript abundance declined rapidly, with a $t_{1/2}$ of 60 minutes (Figure 4B). Elongated HIV-1 mRNA transcripts in TNF-α-pulsed samples were moderately induced in the first hour; however, sustained induction was not observed, and after 2 h the abundance of elongated transcripts declined to less than 1 copy per cell. Hence, transient induction of NF-κB was sufficient to drive limited transcriptional activation of the latent HIV-1 provirus, but this early induction requires sustained NF-κB activity to develop robust expression of full-length HIV-1 mRNA transcripts.

TNF-α induction of HIV-1 Tat synergizes with active NF-κB to promote efficient transcriptional elongation of latent HIV-1 mRNA

The delayed accumulation of elongated HIV-1 mRNA transcripts relative to that of initiated transcripts in J-Lat cells continuously stimulated with TNF-α raised the possibility that efficient elongation may be dependent on *de novo* synthesis of a κB-responsive gene. To investigate this possibility, initiated and elongated HIV-1 transcripts were quantitated in TNF-α stimulated J-Lat 6.3 cells treated with the translational inhibitor cycloheximide. IκBα resynthesis in TNF-α-treated cells was strongly impaired in cycloheximide-treated cells, indicating the effectiveness of this inhibitor (data not shown). Cycloheximide treatment modestly enhanced accumulation of initiated and elongated HIV-1 transcripts in J-Lat cells continuously stimulated with TNF-α for 1 h, indicating that these early transcripts are not dependent on *de novo* protein synthesis (Figure 5A and B). In contrast, the heightened production of elongated HIV-1 transcripts observed in samples treated with TNF-α for 6 h was sharply reduced in the presence of

cycloheximide. These results suggest that NF-κB induction of transcriptional initiation and low-level elongation of HIV-1mRNA do not require *de novo* protein synthesis, however high-efficiency elongation is dependent on newly synthesized proteins in J-Lat cells. The increased levels of initiation induced in cycloheximide-treated cells are likely a consequence of the inhibition of IκBα synthesis, resulting in protracted NF-κB activity. Levels of β-actin mRNA remained unchanged for the duration of the experiment, suggesting cycloheximide-induced toxicity was not a factor in the observed impairment of elongated HIV-1 mRNA transcript accumulation (data not shown).

Expression of Tat, a well-characterized positively acting viral factor-that promotes effective RNA Pol II elongation, is induced following sustained NF-κB activation in HIV-infected cells. Thus we hypothesized that Tat might be the key NF-κB-inducible factor required for high-level transcriptional elongation observed at later time points. To examine whether expression of Tat was sufficient to rescue expression of latent HIV-1 in response to transient NF-κB induction, J-Lat 6.3 cells were transfected with a Tat or control expression vector, pulse or continuously stimulated with TNF-α, and HIV-directed GFP expression was assessed by flow cytometry. Consistent with prior observations, ectopic expression of Tat induced HIV-1 LTR-driven GFP expression in only a minority of cells (Figure 5C). In contrast, Tat-transfected cells pulsed or continuously treated with TNF-α uniformly expressed GFP. Of note, mean GFP fluorescence in Tat-transfected and TNF-α pulsed J-Lat cells was one third of that detected in continuously stimulated cells (data not shown). These observations indicate that the initial wave of NF-κB recruited to the HIV-1 LTR is sufficient to drive effective

gene expression in the context of Tat expression and argue against the notion that functionally different forms of NF-κB recruitment in first and second waves.

To examine whether long-term NF-κB induction could induce efficient latent HIV-1 gene expression in the absence of Tat, we employed A72 cells, Jurkat cells containing a latent HIV-1 LTR driving GFP expression, but lacking the Tat gene. siRNA depletion of Tat was considered, but because the GFP transcript driven by J-Lat cells would contain the mRNA sequence targeted by the necessary siRNA before splicing, this approach was abandoned. J-Lat 6.3 or A72 cells were pulsed or continuously treated with TNF-α, cultured for 16 h, and mean fluorescence expression of GFP in expressing cells was measured by flow cytometry. Pulsed treatment of TNF-α increased mean GFP fluorescence in J-Lat 6.3 cells from a baseline of 14.7 to 18.6, and A72 were similarly increased from 15.6 to 19.2 (Figure 5D). In contrast, continuous TNF-α treatment increased mean GFP fluorescence in J-Lat cells to 227, whereas tat-deficient A72 cells increased to 46.8. These results indicate that Tat represents a key factor mediating the large increase in latent HIV-1 gene expression induced during sustained NF-κB induction.

To confirm a role for NF-κB RelA in TNF-α induction of Tat sensitivity, J6.3κRed cells were nucleofected with siRNA specific for RelA or GL3 as a control. Effective "knockdown" of RelA was confirmed, and the cells were transfected with a Tat or control expression vector, and GFP expression in response to pulsed TNF-α stimulation was assessed by flow cytometry 16 hours later. Pulsed TNF-α stimulation failed to induce significant GFP expression in J-Lat cells transfected with control expression vector in either RelA or GL3 knock down cells. Further, κB-driven DsRed2 expression was

sharply impaired in RelA knockdown cells relative to control GL3 knockdown cells. TNF-α pulse stimulation of Tat-transfected cells strongly induced both GFP and DsRed2 expression in GL3-knockdown cells. However, this response was markedly blunted in RelA knockdown cells. Similar results were observed with J-Lat clones 8.4 and 15.2 (data not shown). These findings indicate that TNF-α-activation of RelA is a necessary cofactor for Tat-induced expression of latent HIV-1.

Impairment of early TNF-α induction of elongated HIV-1 mRNA transcripts from the latent HIV-1 LTR is not a consequence of a defect in RNA polymerase II phosphorylation.

The low level of elongated HIV-1 mRNA transcript accumulation despite rapid synthesis of initiated transcripts suggested a defect in the elongation properties of RNA Pol II. Elongation of HIV-1 mRNA is strongly dependant on phosphorylation of the carboxy-terminal domain of RNA Pol II mediated by P-TEFb (Herrmann and Rice, 1995). RelA may similarly direct P-TEFb to κB-responsive promoters (Barboric et al., 2001). The precise role of NF-κB-mediated recruitment of P-TEFb to the HIV-1 LTR has not been explored. To examine whether early TNF-α induced elongation of HIV-1 mRNA transcripts is P-TEFb dependent or rather is a consequence of "slip-through" elongation of unphosphorylated polymerase complexes, we examined the effect of the P-TEFb inhibitor DRB on Tat-independent early elongation of HIV-1 mRNA transcript formation in cells stimulated with TNF-α for 1 h. As a positive control, DRB inhibition of late Tat-dependent elongation of HIV-1 mRNA in samples treated with TNF-α for 6 h was also assessed. TNF-α stimulation for 6 hours induced accumulation of ~500 initiated

and ~300 elongated transcripts per cell (Figure 6A and B). DRB strongly interfered with elongated transcript formation in these samples, reducing transcript abundance to an undetectable level. However, initiated transcript formation was similar to samples not treated with DRB. Samples treated with TNF-α for 1 h amassed ~10 elongated transcripts per cell and ~120 initiated transcripts. DRB treatment had little effect on the accumulation of initiated HIV-1 mRNA transcripts in samples treated for 1 h while elongated transcript formation was markedly reduced to levels below the limits of detection (Figure 6A and B). These results indicate that both early Tat-independent and late Tat-dependent TNF-α induced elongation relies on active P-TEFb-dependent phosphorylation of RNA Pol II and suggest that P-TEFb is recruited to the HIV-1 LTR shortly after TNF-α stimulation.

Although early TNF-α induced elongation of HIV-1 mRNA transcripts is apparently P-TEFb dependent, RelA-recruited P-TEFb still might function as a less efficient RNA Pol II kinase than late Tat-recruited P-TEFb. To assess the kinetics of P-TEFb kinase activity at the HIV-1 LTR induced by TNF-α, ChIP analysis of phospho-RNA Pol II was performed in J-Lat samples treated with TNF-α for various times. Phospho-Ser2-RNA Pol II immunoprecipitates were enriched in HIV-1 LTR DNA from extracts treated with TNF-α for 15 and 30 min (Figure 6C). However, this enrichment was reduced in extracts of cells stimulated for 1 hour, consistent with the reduced overall level of RNA Pol II associated with the HIV-1 LTR. Extracts of cells treated for 2, 4, and 6 h with TNF-α were enriched in HIV-LTR DNA to similar levels as observed in 15- and 30-min samples. These findings suggest that the kinase complex driving phosphorylation of RNA Pol II on the HIV-1 LTR has similar activities during both the early and late phases

of HIV transcription. Consequently, the inefficiency of elongation in early TNF-α-induced HIV-1 mRNA transcription is unlikely to be solely a consequence of insufficiently localized P-TEFb kinase activity.

Discussion-

To further delineate the role of NF-κB in the activation of latent HIV-1 proviruses, we performed a kinetic analysis of RelA and RNA polymerase II interaction with the LTR in the J-Lat model of HIV-1 latency. We observed an unexpectedly dynamic oscillating pattern of RNA Pol II recruitment to the LTR driven by the inherent damped oscillatory nature of NF-κB activation driven by NF-κB-dependent activation of IκBα and perhaps A20. Two recent studies have demonstrated that sustained induction of the NF-κB signaling pathway produces similar oscillations of RelA and RNA Pol II at various host gene promoters (Covert et al., 2005; Werner et al., 2005), and concluded that these effects reflect the activation of IκBα and A20 gene expression. The pattern of RelA and RNA Pol II recruitment to the HIV-1 LTR is coincident with the pattern of overall nuclear enrichment of RelA in response to TNF-α stimulus, implying that LTR binding is reflective of general NF-κB abundance. Indeed, when NF-κB signaling was limited to a single round by transient stimulation (15 min) with TNF-α, the oscillatory nature of RelA and RNA polymerase II recruitment to the latent HIV-1 LTR was lost.

Transient induction of NF-κB is sufficient to induce the synthesis of many κB-responsive genes, most notably IκBα (Sun et al., 1993). Consequently it was unexpected that NF-κB dependent activation of latent HIV-1 would require sustained NF-κB induction. To confirm that general κB-responsive gene expression is inducible following

transient induction of NF-κB, we constructed new Jurkat and J-Lat based cell lines containing integrated κB-DsRed2 reporter genes. Analysis of these cell lines provided strong evidence that transient induction of NF-κB is sufficient for generalized κB-dependent gene expression, however not for effective expression of latent HIV. The features of the HIV-1 LTR that are responsible for this difference remained unclear.

Analysis of HIV-1 mRNA transcript formation immediately after induction of NF-κB revealed that, while initiated transcripts amass rapidly, transcriptional elongation is impaired. Consistent with this observation, early studies of HIV transcription described NF-κB as a strong promoter of transcriptional initiation (Nabel and Baltimore, 1987). More recent studies of NF-κB in HIV transcription suggest it also plays a major role in transcription elongation in addition to its role as an initiation factor (West et al., 2001). Low level accumulation of elongated HIV mRNA transcripts are apparent immediately after TNF-α stimulation. However, the rate of accumulation of these elongated transcripts is greatly reduced relative to the rate of initiated transcript synthesis. The rate of elongated transcript formation begins to approximate the rate of initiated transcript formation only after 4-6 h of cellular stimulation. This observation suggests that the initial round of NF-κB recruitment to the HIV-1 LTR may promote inefficient elongation which is sufficient to produce small quantities of Tat. This newly synthesized Tat then synergizes with subsequent rounds of transcriptional initiation induced by NF-κB to promote highly efficient elongation. This hypothesis is supported by the observed dependence of late efficient elongation on *de novo* protein synthesis (Tat must be synthesized). Additionally, the observation that ectopic expression of Tat drives robust expression of latent HIV-1 in response to transient induction of NF-κB suggests that the

absence of this transactivator is key to this failure of induced gene expression. A recent computational study of Tat and HIV expression further supports this conclusion (Weinberger et al., 2005).

The ability of NF-κB to induce transcriptional elongation has been ascribed to its ability to recruit P-TEFb, the same RNA Pol II kinase partner used by Tat to induce elongation (Barboric et al., 2001). Our studies with the P-TEFb inhibitor DRB indicate that early TNF-α driven, NF-κB-dependent transcriptional elongation is impaired in the presence of DRB. Further assessment of functional phosphorylation of RNA Pol II with anti-phospho-serine-2-RNA Pol II ChIP analyses confirm that the polymerase complex is similarly phosphorylated in both early and late TNF-α stimulated samples, suggesting that recruitment of NF-κB is sufficient to induce this modification. As both NF-κB and Tat appear to similarly employ P-TEFb to induce transcriptional elongation, it remains unclear why late Tat-dependent elongation is so much more efficient than early NF-κB dependent elongation. One explanation might be that RNA Pol II phosphorylation is dynamic, and that Tat, which moves with the polymerase complex, induces continuous phosphorylation of RNA Pol II. Alternatively, Tat may recruit factors in addition to P-TEFb that strengthen its function as an elongation factor. Finally, Tat may direct P-TEFb to more efficiently phosphorylate factors aside from RNA Pol II, such as the inhibitory DSIF or NELF proteins that negatively regulate RNA Pol II. The strong synergy observed between Tat and NF-κB inducing stimuli in the activation of HIV transcription further supports unique activating properties of NF-κB and Tat.

These studies were conducted using the J-Lat model of HIV latency, a model which though representative of HIV-1 latency as it exists *in vivo* may diverge from the natural

state of the latently infected cell. Unfortunately, systematic molecular and biochemical studies of latently infected cells isolated from patients is not possible due to an inability to purify these rare cells. However, NF-κB has been shown to exhibit a damped oscillatory pattern of induction within stimulated primary T-cells (Covert et al., 2005; Werner et al., 2005).

The finding that sustained induction of NF-κB is required for κB-driven activation of HIV latency may have implications for therapeutic interventions aimed at eliminating this viral reservoir. The design of potential activators should be selected and employed in a manner that sustains the induction of NF-κB. Alternatively, these findings support consideration of combined application of sTat and transient agonists of NF-κB. Given the broad assortment of host genes induced by NF-κB, this latter approach might avoid the toxicity likely to be associated with sustained systemic induction of NF-κB.

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Figure 1. TNF-α stimulation induces bimodal association of RNA polymerase II with the latent HIV-1 LTR. **(A)** TNF-α stimulation drives bimodal, synchronous recruitment of RNA Pol II and RelA to the latent HIV-1 LTR. Fixed chromatin extracts from J-Lat 6.3 cells treated with 20 ng/ml TNF-α for various times or left untreated were immunoprecipitated with antibodies specific for RelA, RNA Pol II, or without antibody as a non-specific control. Immunoprecipitates were assessed for enrichment in HIV LTR DNA by UV visualization of PCR products in an ethidium bromide-stained gel. Data are representative of three separate experiments. Note the synchronicity of RNA Pol II and RelA recruitment to the HIV-1 LTR, as well as the nadir in binding 1 hour after TNF-α treatment. **(B)** TNF-α-induced recruitment of RelA and RNA Pol II to the latent HIV-1 LTR coincides with IκBα degradation. Cytoplasmic extracts of samples treated as in (A) were prepared, and IκBα levels were assessed by immunoblot analysis. Cytoplasmic RelA levels were assessed to confirm equivalent loading of samples. **(C)** TNF-α induces bimodal nuclear enrichment of RelA. Nuclear extracts of samples treated as in (A) were prepared and analyzed for κB-DNA binding activity (upper panel) or for RelA enrichment by immunoblot. Nuclear HDAC1 levels were assessed to confirm equivalent loading. Note the largely overlapping patterns of RelA nuclear enrichment and its recruitment to the latent HIV-1 LTR.

Figure 2. Transient induction of NF-κB induces unimodal recruitment of RNA polymerase II to the latent HIV-1 LTR. **(A)** Pulsed administration of TNF-α induces IκBα degradation. J-Lat 6.3 cells were stimulated with 20 ng/ml TNF-α or left untreated for 15 min, washed twice in medium, and returned to culture for various times.

Cytoplasmic extracts were prepared, and IκBα levels were assessed by immunoblot. Cytoplasmic RelA levels were assessed to confirm equivalent loading of samples. Note the similarity in depletion of IκBα in transiently and continuously TNF-α treated samples (1B). **(B)** Pulsed administration of TNF-α induces transient activation of NF-κB. Nuclear extracts of samples treated as in (A) were analyzed for κB-DNA binding activity (upper panel) or for RelA enrichment by immunoblot. Nuclear HDAC1 levels were assessed to confirm equivalent loading. **(C)** Pulsed TNF-α administration induces a unimodal pattern of RelA and RNA polymerase II recruitment to the latent HIV-1 LTR. Fixed chromatin extracts of samples treated as in (A) were subjected to immunoprecipitation with antibodies specific to RelA, RNA Pol II, or without antibody as a non-specific control. Immunoprecipitates were assessed for enrichment in HIV LTR DNA by UV visualization of PCR products in an ethidium bromide stained gel. Data are representative of three separate experiments. Note the absence of a second wave of RNA Pol II recruitment to the latent HIV-1 LTR.

Figure 3. Transient induction of NF-κB is sufficient to induce robust general κB-dependant, but not latent HIV-1 gene expression. **(A)** Transient TNF-α administration induces poor expression of latent HIV-1. J-Lat 6.3 cells were left untreated or stimulated with 20 ng/ml TNF-α continuously or for 15 minutes followed by washing and continued culture. HIV-LTR dependent expression of GFP was assessed by flow cytometry. Note the overall lack of GFP expression in samples transiently treated with TNF-α. **(B)** Transient induction of NF-κB is sufficient to drive general κB-dependent gene expression. JκRed cells were treated as in (A), and κB-dependent expression of DsRed2

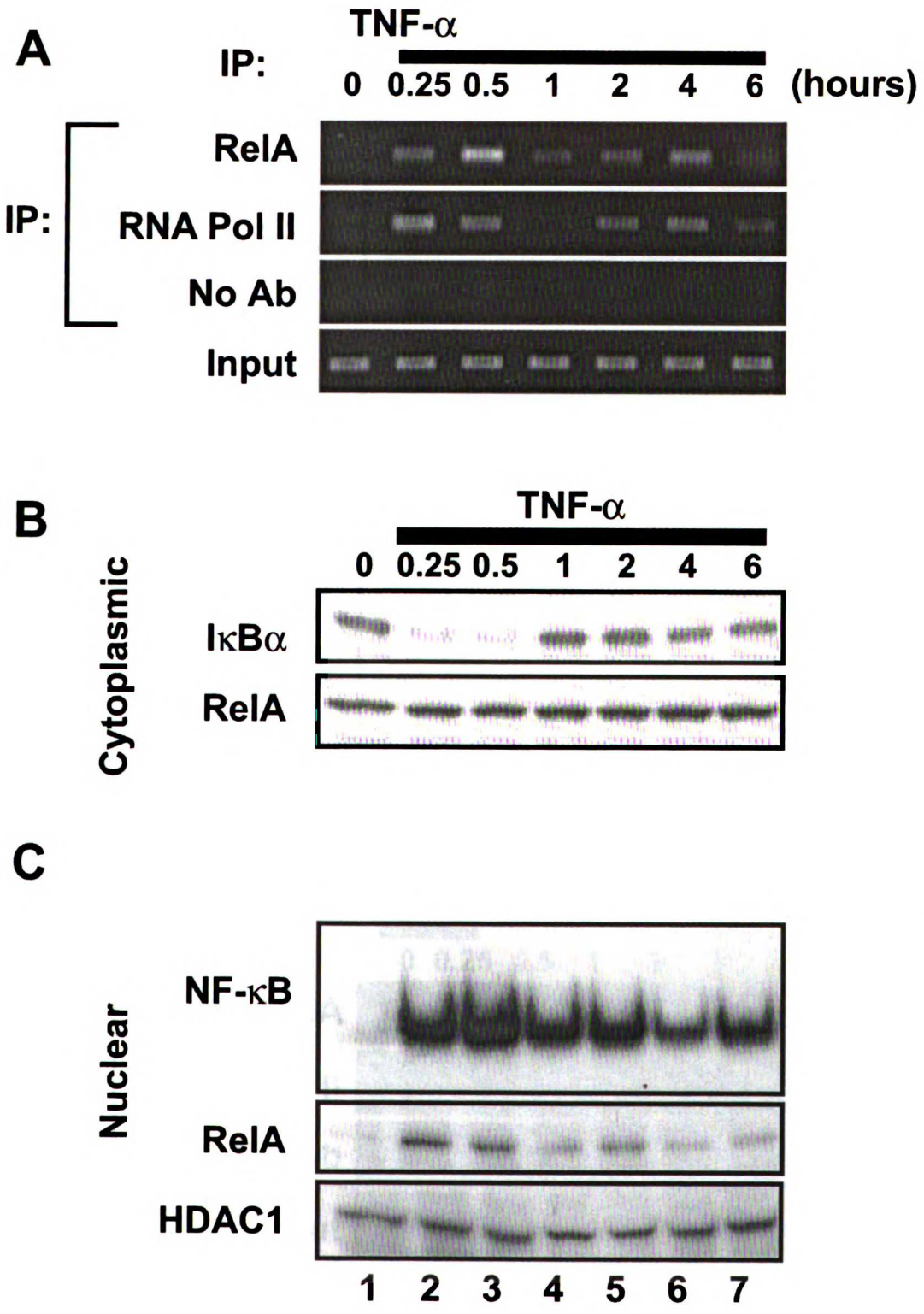
was assessed by flow cytometry. Note the strong induction of κB-dependent gene expression by transient TNF-α stimulation. **(C)** Transient NF-κB induction induces robust expression of κB-dependent genes, but not latent HIV in J-Lat 6.3 cells. J6.3κRed cells were treated as in (A), and HIV-1 LTR-dependent expression of GFP, and κB-dependent expression of DsRed2 were assessed by flow cytometry. Note the strong induction of κB-dependant gene expression and relative absence of HIV gene expression induced by transient TNF-α stimulation.

Figure 4. TNF-α induces delayed efficient elongation of HIV mRNA transcripts in J-Lat cells. **(A)** TNF-α treatment of J-Lat cells induces rapid accumulation of initiated, but not elongated HIV-1 mRNA transcripts. J-Lat 6.3 cells were treated with 20 ng/ml TNF-α for the times indicated, and total RNA was extracted. Initiated and elongated HIV mRNA transcripts were quantitated by real-time RT-PCR. Note the delayed emergence of elongated HIV transcripts relative to the rapid increase in initiated transcripts. **(B)** Transient induction of NF-κB does not induce accumulation of elongated HIV mRNA transcripts. J-Lat 6.3 cells were treated with 20 ng/ml TNF-α for 15 min, washed twice, and returned to culture for various times. Initiated and elongated HIV mRNA transcript abundance were assessed as in (A). Note the absence of delayed elongated HIV mRNA transcript accumulation relative to (A). **(C)** The kinetics of initiated and elongated HIV transcript formation in TNF-α induced J-Lat cells are dynamic. Rate of transcript formation in continuously TNF-α stimulated J-Lat 6.3 cells was determined from (A). Note that rate of initiated HIV mRNA transcript formation is relatively constant across time, in contrast to the accelerating rate of elongated transcript formation.

Figure 5. TNF-α induced robust expression of HIV is dependent on *de novo* synthesis of Tat. **(A)** TNF-α induced accumulation of initiated HIV mRNA transcripts is not dependent on *de novo* protein synthesis. J-Lat 6.3 cells were preincubated with 10 μg/ml cycloheximide for 30 min or left in complete culture medium prior to 15-min pulse or continuous stimulation with TNF-α for 1 or 6 h. Total RNA was extracted and initiated HIV mRNA transcripts were quantitated by real-time RT-PCR. Note the continued accumulation of initiated HIV transcripts in cycloheximide-treated samples at both 1 and 6 h after TNF-α stimulation. Data are representative of three separate experiments. **(B)** Late TNF-α induced accumulation of elongated HIV mRNA transcript is dependent on *de novo* protein synthesis. Elongated HIV-1 mRNA transcripts were quantitated from samples treated as in (A). Note the sensitivity of elongated HIV-1 mRNA transcript accumulation in cells continuously stimulated with TNF-α to cycloheximide treatment. Data are representative of three separate experiments. **(C)** Ectopic expression of HIV-1 Tat rescues HIV gene expression in response to transient TNF-α stimulus. J-Lat 6.3 cells were cotransfected with control empty CMV, Tat, or RelA expression vectors and a plasmid expressing the cell-surface H-2K^k marker to identify transfected cells. Transfected cells were stimulated with TNF-α for 15 minutes or continuously, and GFP expression was assessed in the H-2K^k-expressing cells. **(D)** Continuous TNF-α stimulation of A72 cells drives weak expression of HIV. Tat-deficient A72 cells were left untreated, or pulsed or continuously treated with TNF-α, and GFP expression was analyzed by flow cytometry.

Figure 6. NF-κB-mediated induction of latent HIV-1 gene expression is DRB-sensitive. **(A)** TNF-α induced initiation of HIV transcription is DRB insensitive. J-Lat 6.3 cells were left untreated or pretreated with DRB for 30 min, then left unstimulated or stimulated with 20 ng/ml TNF-α for 1 or 6 h. Total RNA was extracted, and initiated HIV mRNA transcripts were quantitated by real-time RT-PCR. **(B)** Early and late TNF-α induced HIV mRNA transcript elongation is DRB sensitive. Elongated HIV mRNA transcripts were quantitated from samples treated as in (A). Note the strong sensitivity of elongated transcript formation at both 1 and 6 h after TNF-α stimulation. **(C)** TNF-α stimulation induces rapid recruitment of phosphorylated RNA Pol II to the HIV-1 LTR. Fixed chromatin extracts were prepared from J-Lat 6.3 cells stimulated with 20 ng/ml TNF-α continuously or transiently for 15 minutes and cultured for various times. Extracts were immunoprecipitated with antibodies specific for serine-2 phosphorylated RNA polymerase II and assessed for enrichment in HIV LTR DNA by UV visualization of PCR products in an ethidium bromide-stained gel. Data are representative of three separate experiments. Note the presence of phosphorylated RNA Pol II on the HIV LTR in samples stimulated for 15 min.

Williams_Figure 1



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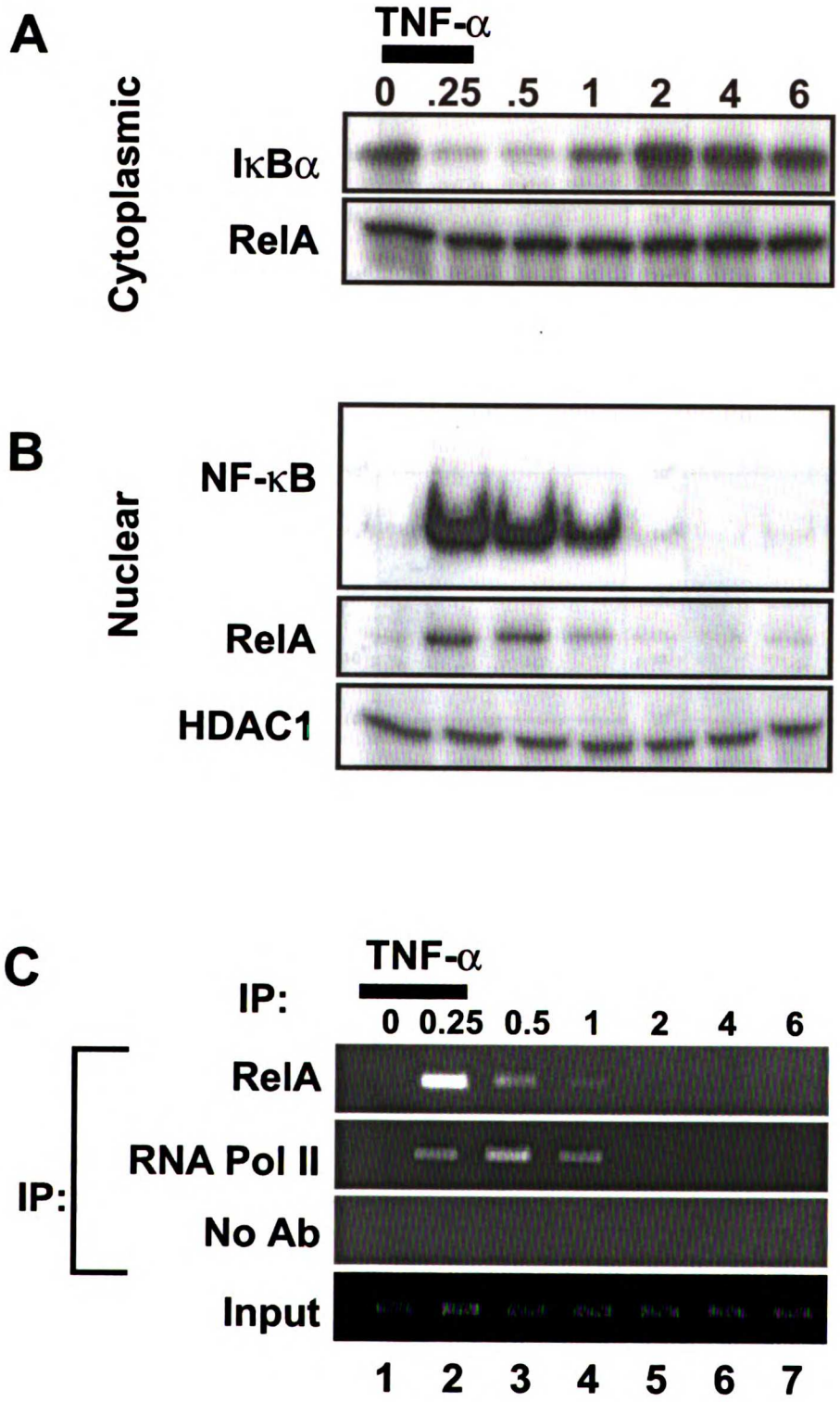

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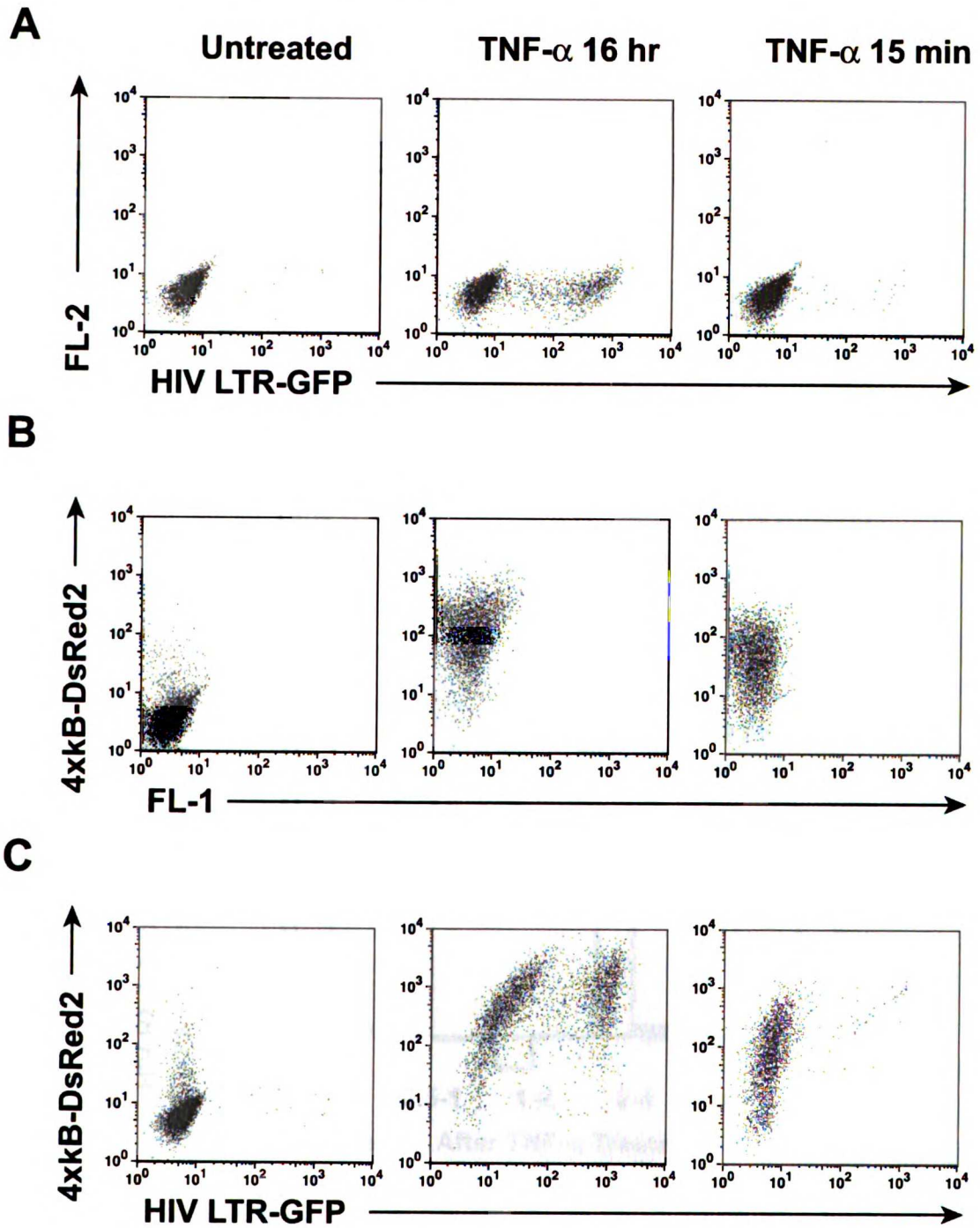

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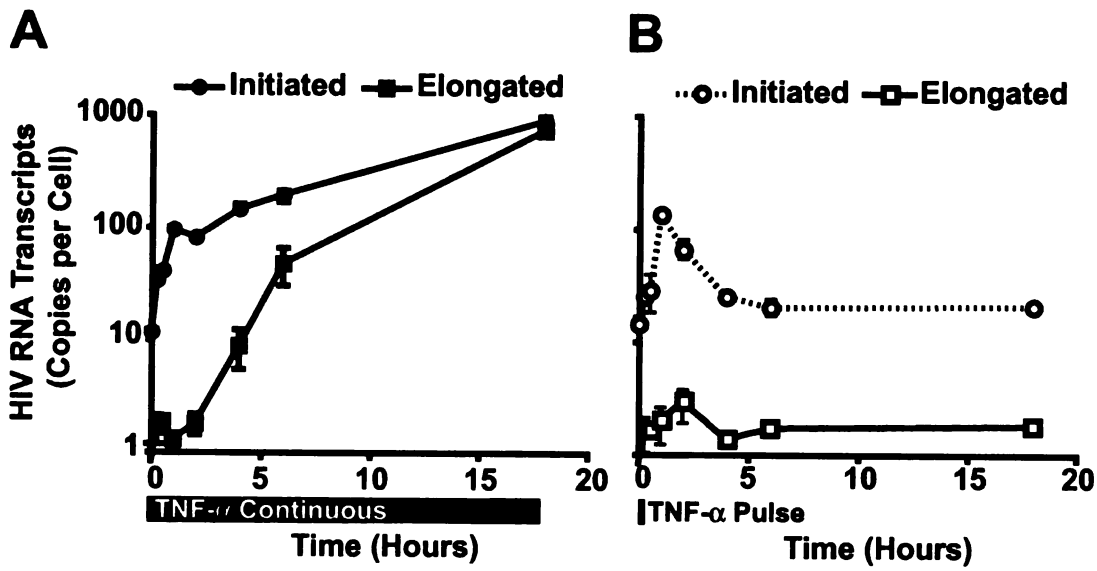

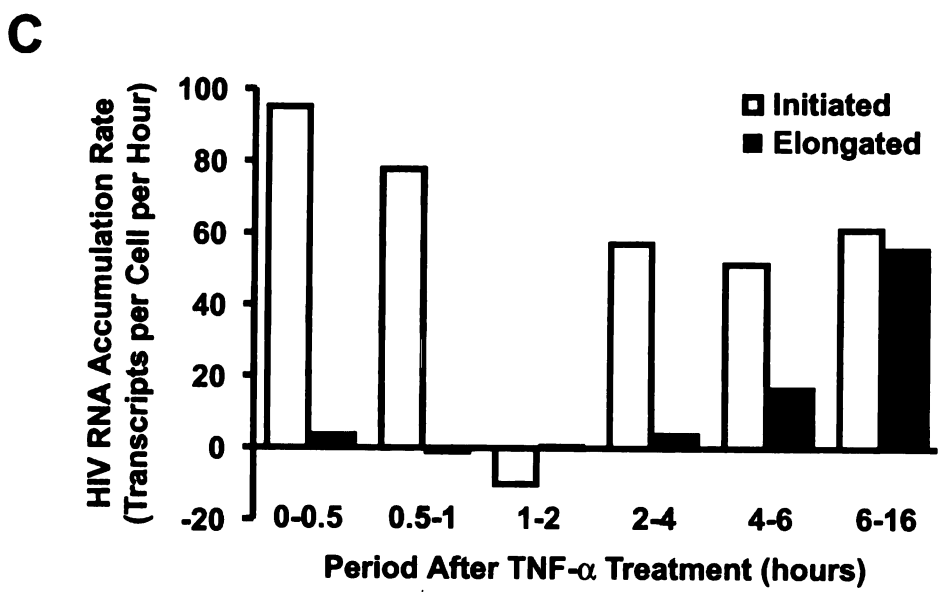

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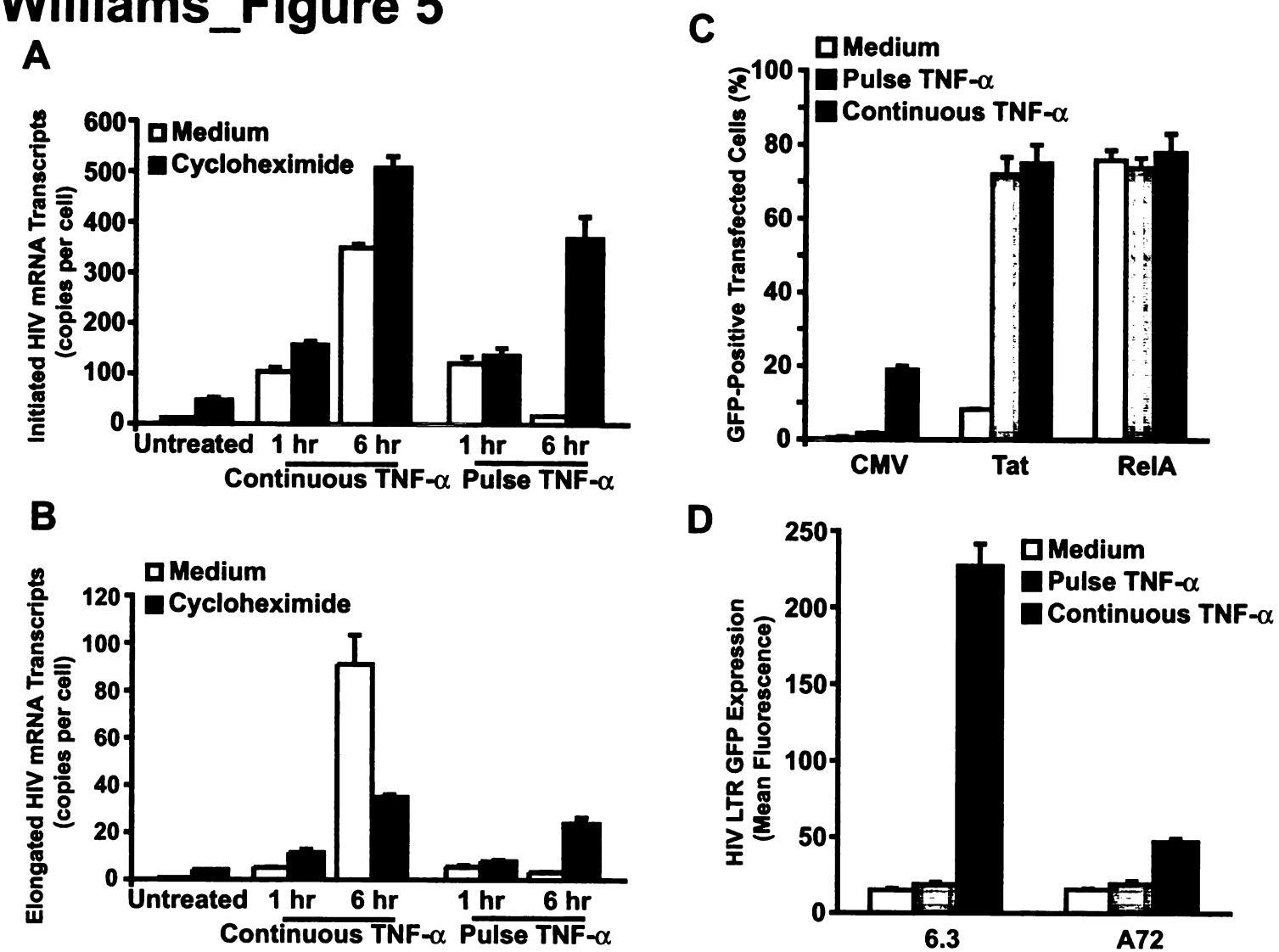

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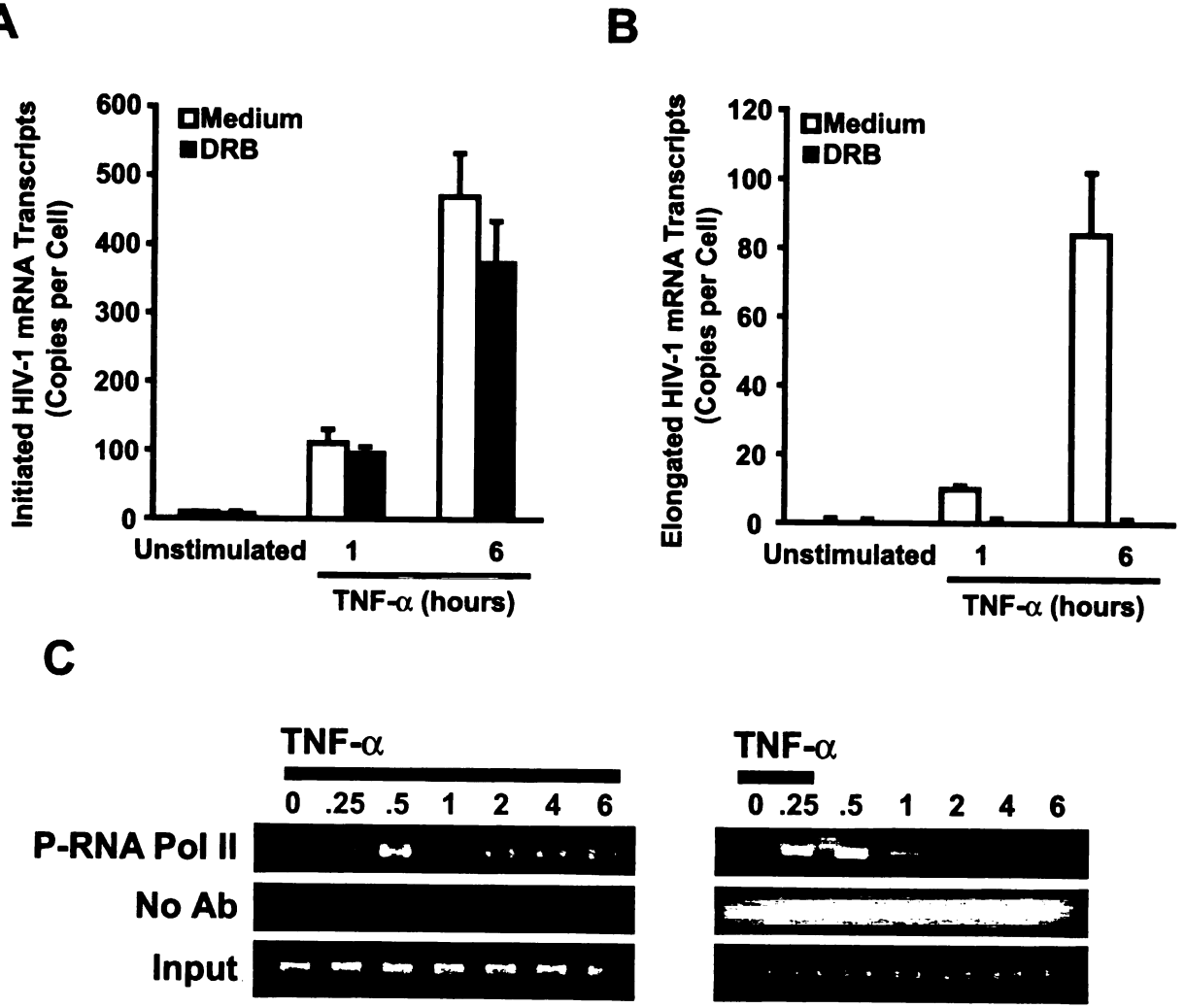

Chapter 5

Conclusions

A broad understanding of the mechanisms that maintain and promote resolution of HIV-1 latency will be key to developing therapies designed to eradicate this resistant reservoir of infection. In my thesis research, I identified different members of the NF-κB family of transcription factors as key regulators of both activation and maintenance of HIV-1 latency and developed insights into the basic transcriptional events surrounding the persistence of latent HIV proviruses and their subsequent activation. Insights gleaned from these studies suggest new approaches aimed at purging HIV from its latent reservoir. These planned studies take the form of small clinical trials that will be performed in both infected patients and in rhesus macaques where a model of SIV latency has been developed with our collaborators at the University of California, Davis.

I. Prostratin induction of NF-κB activates expression of latent HIV-1

The recognition of a pool of latent HIV-1 in infected patients as an important reservoir of HAART-insensitive virus prompted investigation into potential therapeutic remedies. Early efforts with broadly immunoactivating therapies, including general T-cell activation with OKT3 antibodies or IL-2 cytokine stimulation, were clinically unsuccessful [1, 2]. These efforts failed in part due to the severe side effects associated with the use of such broadly activating therapies. Consequently, new efforts were undertaken to identify more tolerable antagonists of HIV-1 latency.

Prostratin, the active component of a traditional Samoan herbal remedy administered for a range of different diseases including yellow fever, was identified as a potent activator of HIV gene expression [3]. In view of its apparent tolerability in ethnobiological settings, interest in this compound as an anti-HIV latency compound

began to accrue. Prostratin is a non-tumorigenic phorbol ester, a class of molecules with strong HIV-inducing activity. Investigation in a SCID-HU derived model of HIV latency revealed that prostratin could induce expression of latent HIV-1 [4], a finding further supported by the analysis of prostratin effects on HIV-1 expression in blood cells from infected patients [5]. These studies, while suggestive of an effect in latently infected cells, did not exclude the possibility that prostratin amplified expression of HIV-1 in actively infected cells. To address this question directly, we examined the effects of prostratin in the J-Lat T-cell model of HIV-1 latency, in which true HIV-1 latency is confirmed in individual cells by the absence of HIV-1-directed expression of GFP. Our studies confirmed that prostratin is a potent antagonist of HIV-1 latency, inducing efficient expression of latent HIV-1 in a subset of latently infected cells. Induction of latent HIV-1 gene expression by prostratin was observed in multiple J-Lat clones, indicating that the activity of prostratin occurs independent of the locus of HIV integration.

We identified the PKC family of signal-transducing protein kinases as key partners in prostratin induction of HIV-1 gene expression. In this manner, prostratin is functions in a highly analogous manner to the tumorigenic phorbol ester PMA, which similarly induces activation of AP-1 and NF-κB [6, 7]. Analysis of upstream PKC signaling events revealed similarity in the range of kinases induced by these phorbol esters; however, PMA induced membrane translocation of PKC-α and PKC-ζ much faster and more strongly than prostratin. This distinction may provide clues to the sharply different tumor-inducing profiles of these two compounds. Our analyses concluded that prostratin action requires the novel family of PKC kinases. A subsequent

study confirmed our observations and identified sequential action of the novel PKCs PKC-α and PKC-θ as the individual kinase mediators of prostratin's action on the latent HIV-1 provirus [8]. This later study made use of siRNA-mediated targeted knockdown of host gene expression, a technology that was not yet refined at the time of our own study. Further study of targeted PKC-α-specific inducers may yield potent and systemically tolerable anti-latency agents. Additionally, knockdown of individual PKC subunits may identify those members responsible for PMA-induced carcinogenesis.

We further evaluated the AP-1 and NF-κB signaling pathways downstream of the novel PKCs as intermediaries of prostratin antagonism of HIV-1 latency. These families of transcription factors had been implicated in prior studies as positive regulators of activated HIV-1 gene expression, but not in the setting of latency [9, 10]. Our studies of HIV-1 LTR reporters with targeted mutations in various enhancer sites demonstrated that the NF-κB binding sites are essential for prostratin induction of HIV-1 gene expression. In contrast, the AP-1 sites appear dispensable for prostratin action on the HIV-1 LTR. These findings are consistent with earlier studies that identified substantial replicative defects in viruses with mutated κB enhancer binding sites [11]. A series of investigations with chemical and genetic inhibitors of NF-κB further confirmed the requirement for NF-κB activation in prostratin antagonism of HIV-1 latency.

We further identified the key role played by NF-κB by demonstrating that RelA directly binds to the latent HIV-1 LTR in chromatin immunoprecipitation assays. Before these studies, binding of this transcription factor to the HIV-1 LTR had been predicted by in vitro electrophoretic mobility shift assays; however, direct binding in vivo had not been demonstrated. Our analyses conclusively established inducible binding of RelA to

the HIV-1 promoter, as well as constitutive binding of Sp1 at an adjacent enhancer site. These studies identified conditions for ChIP analysis of transcription factor binding to the latent HIV-1 LTR in J-Lat cells, and laid the groundwork for the subsequent study of p50 homodimer-mediated repression of latent HIV transcriptional initiation (Chapter 4). These findings regarding NF-κB were subsequently confirmed by another laboratory [12].

II. NF-κB1 p50 Homodimers Bind to, and Transcriptionally Repress Latent HIV-1

In our initial analysis of factors binding to the latent HIV-1 LTR, the constitutive binding of p50 subunit of NF-κB was a striking feature. Earlier analyses of chromatin-integrated HIV-1 indicated that the κB loci of the HIV-1 LTR are protected against micrococcal nuclease digestion, suggesting binding of an unknown factor [13, 14]. NF-κB1 p50 homodimers represent the predominant NF-κB species in the nuclei of uninduced cells [15, 16], and the primary candidate to bind the κB enhancer of the HIV LTR in uninduced cells. We directly demonstrated that this factor is associated with the transcriptionally silent HIV-1 LTR in J-Lat cells. Strikingly, the quantity of p50 associated with the HIV-1 LTR is similar in uninduced and TNF-α-induced cells. This observation is explained by a model in which the p50-p50 homodimer is replaced by a p50-RelA heterodimer upon induction of NF-κB. Since p50 is contained in either LTR-binding NF-κB complex, the ChIP assay does not identify a change in p50 binding. Alternatively, the amount of p50 associated with the HIV-1 LTR may change, but not enough to be detected by the ChIP assay.

The prevailing model of HIV-1 transcriptional regulation states that RNA polymerase II binds the LTR constitutively and initiates transcription that is abortive in the absence of Tat or other T-cell activating stimuli [17]. In contrast to this model, we failed to detect binding of RNA Pol II to the latent HIV-1 LTR in the absence of NF-κB activating stimulus. This observation suggested that the failure of gene expression in J-Lat cells is not a consequence of abortive elongation, but is rather a failure of the earlier step of transcriptional initiation. Restriction of transcriptional initiation is a common mechanism for regulating gene expression. Therefore, the processes involved in the restriction of HIV-1 initiation in latently infected cells may be common to processes utilized to restrict expression of endogenous host genes. NF-κB1/p50 has been demonstrated to repress expression of the host genes IL-8 and Cox-2, in the absence of NF-κB activating stimulus [18]. Our studies revealed p50-complexes as transcriptional repressors of latent HIV, and highlighted the yin and yang functions of different Rel proteins in the maintenance (p50 homodimers) and loss (p50/RelA heterodimers) of HIV latency.

Histone acetylation is a common indicator of transcriptional activity [19]. Our studies revealed that histones surrounding the latent HIV-1 LTR are hypoacetylated, a process directed by the localized recruitment of various histone deacetylases (HDACs). Induction of latent HIV-1 gene expression is associated with an increase in localized histone acetylation. As such, we reasoned that this histone modification might underlie the transcriptional processes restricting RNA polymerase II association with the latent HIV-1 LTR. Studies by other groups had confirmed an increase in histone acetylation after NF-κB induction of HIV gene expression [12]. We next demonstrated a functional

role for histone acetylation in the context of HIV-1 latency. Induction of generalized histone hyperacetylation with the use of HDAC inhibitor TSA triggered the association of RNA polymerase II with the latent LTR in the J-Lat system, but failed to induce gene expression. We showed this to be a consequence of a failure of TSA to induce C-terminal domain phosphorylation of RNA polymerase II, a necessary modification for elongation by this bound polymerase complex.

We conclude that transcriptional regulation of latent HIV-1 can be governed both at the level of transcriptional initiation through HDAC-mediated exclusion of the RNA polymerase complex and at the level of mRNA elongation as predicted by the canonical model of HIV transcriptional regulation. Thus, our observations do not contradict the established model. Rather, they indicate that HIV latency in some contexts involves an additional level regulation involving blockade of RNA polymerase II binding. Other systems examining TSA action have observed that inhibition of HDAC activity alone is sometimes sufficient to induce expression of HIV [13, 20]. It is not clear whether the action of TSA in these systems is due solely to a reversal of repression of transcriptional initiation, or rather to the enhancement of activity NF-κB complexes which are positively regulated by acetylation [21, 22].

Several transcription factors, including p53 and NF-κB, are modulated by post-translational acetylation [21, 23]. Acetylation of RelA drives prolonged nuclear duration of the complex and enhances its transcriptional activity. Studies with the J-Lat system identified a strong synergy between NF-κB-inducing agents and TSA in the induction of HIV-1 gene expression. The synergy observed with these combined treatments may be a consequence of enhanced NF-κB activity, relaxed chromatin structure, or a combination

of both factors. Alternatively, unrecognized partners may be induced by simultaneous treatment that enhance transcriptional activation of latent HIV through alternate mechanisms.

Overall activation of HIV gene expression by NF-κB-inducing stimuli was modestly reduced in p50-knockdown cells, suggesting that the p50/RelA heterodimer is a more potent activator of transcription than RelA/RelA homodimers. Prior studies of NF-κB transactivation indicate that the p50/RelA heterodimer binds DNA with greater affinity than RelA/Rel homodimers, providing a potential explanation for this observation.

The development of tools to specifically target downregulation of p50 will allow a broader assessment of this mechanism in the regulation of host genes. A study of the basal expression of host κB-responsive genes may identify subsets that are repressed by this complex and others that are not. Examination of these subsets might identify DNA sequences and neighboring transcription factors that contribute to the effect. Additionally, the extent of NF-κB2/p52's involvement in the regulation of latent HIV-1 gene expression remains unexplored. Additional derepression might be achieved in the context of synchronous knockdown of p50 and p52.

Our observations in the J-Lat system suggest that inhibition of HDAC action will strongly prime latently infected cells for transcriptional activation in the context of either NF-κB inducers or exogenously supplied Tat. In a recent clinical study in HIV-1-infected patients, the HDAC inhibitor valproic acid transiently reduced the latent pool [24]. Our studies predict that this effect would be strongly enhanced by simultaneous

administration of NF-κB inducing stimuli or soluble transducing Tat. Future studies examining combined therapeutic approaches will be of great interest.

III. Sustained Activation of NF-κB Is Required for NF-κB Induction of Latent HIV-1 Gene Expression

To expand on the observed activation of latent HIV-1 gene expression by NF-κB-inducing stimuli, we conducted an analysis of the kinetics of transcriptional events after NF-κB induction. We observed a surprisingly complex pattern of RNA polymerase II binding to the HIV-1 LTR after induction of NF-κB, with distinguishable waves of recruitment of RNA polymerase II to the HIV LTR. These waves of polymerase recruitment were largely synchronous with a similar pattern of RelA recruitment to the latent HIV-1 LTR. This damped oscillatory pattern is governed by the negative feedback loop inherent to NF-κB, directed through the κB-driven expression of NF-κB inhibitors IκBα and A20 [25, 26].

While transient activation of NF-κB can induce a broad range of κB-responsive genes, several host genes, including MCP-1 and SOD-2, require prolonged induction for efficient expression [27]. Analysis of NF-κB induction of HIV-1 gene expression revealed a striking requirement for prolonged NF-κB induction. Transient induction of NF-κB was capable of inducing transcriptional initiation; however, elongation before 4 hours of NF-κB induction was inefficient. The delayed enhanced elongation is not a consequence of NF-κB directly, but rather a result of de novo synthesis of Tat, which synergizes with continued NF-κB induction to promote efficient elongation.

Interestingly, although transient stimulation of NF-κB induces Tat synthesis, this Tat is not capable of transactivating the HIV LTR in the absence of continued NF-κB activity. This is likely the consequence of rapid re-repression of the HIV LTR after resolution of the NF-κB response. This re-repression is unlikely to be mediated by p50, as p50-knockdown cells are no more sensitive to transient NF-κB induction than control cells (data not shown). The nature of this repression is not yet clear; however preliminary data suggest that histone acetylation is involved. YY1, a transcription factor that bind the LTR, can recruit HDAC1 to the HIV LTR. This complex may recruit deacetylase activity to the LTR in the absence of p50. Additional investigation into this possibility is warranted.

In view of recent reports demonstrating RelA-directed recruitment of p-TEFb, the failure of NF-κB to direct efficient elongation was unexpected [28]. Given this functional homology to Tat and the well-established role of p-TEFb in directing HIV elongation, one might predict that RelA drives strong elongation of the HIV-1 LTR. The observed serine-2 phosphorylation of RNA polymerase II immediately after NF-κB induction in J-Lat cells (i.e. Tat-independent) suggests that P-TEFb kinase activity is recruited to the LTR. The similar intensity of phospho-RNA polymerase II at later time points (i.e. Tat-dependent), indicates that the P-TEFb kinase is similarly active in both conditions. Based on these observations, factors other than P-TEFb may be key to the differential elongating effects observed with NF-κB- and Tat-dependent elongation of HIV-1 RNA transcripts. In a prior in vitro study of NF-κB-induced elongation of HIV transcripts, CDK9 was largely dispensable for RelA transactivation [29]. Thus, the

CDK7 and CDK8 kinases may be more important in this context or, alternatively, functional redundancy may exist within these various assay systems.

A number of possibilities underlying this differential effect remain to be excluded. The chromatin immunoprecipitation assay used to assess serine-2 phosphorylation may not be sensitive enough to detect the degree of phosphorylation of a single RNA polymerase II complex. The C-terminal domain of RNA Pol II, which is the substrate for serine-2 phosphorylation, is comprised of 26 repeats. Hence, NF-κB driven P-TEFb may phosphorylate eight residues, and Tat-driven P-TEFb might phosphorylate all 26. Such a difference might not be distinguished in ChIP assays performed with phospho-serine-2 antibodies. Alternatively, Tat transits with the RNA Pol II complex as it elongates and may be able to promote renewed RNA Pol II phosphorylation as the RNA chain extends, continuously reinforcing its elongating capacity. Tat might also induce more efficient phosphorylation of the negative transcription factors associated with paused RNA polymerase II complexes, NELF, and DSIF. It is also possible that Tat may recruit factors in addition to P-TEFb that synergize to promote highly efficient elongation. These and other events may underlie the dramatic differences observed with NF-κB- and Tat-driven elongation of the RNA polymerase II complex engaged on the HIV LTR.

Although NF-κB appears to induce elongation of HIV-1 mRNA poorly, it directs expression of general κB-responsive genes with great efficiency. Our studies with integrated κB-DsRed2 reporters indicated that transient induction of NF-κB is sufficient for strong expression of this reporter construct. Additionally, expression of κB-responsive host genes IκBα, A20, COX-2, IL-6, and GRO-1 are all strongly and rapidly induced by transient NF-κB induction [27]. The features common to the promoters that

allow effective NF-κB driven expression or those that restrict it in the context of HIV are unclear. Further investigation into promoter and RNA elements that regulate transcriptional events will enhance our understanding of these basic events surrounding the transactivation of gene expression by NF-κB.

IV. Future Directions

This body of work has elucidated the role of the NF-κB family of transcription factors in the regulation of latent HIV-1 gene expression. The primary focus of these studies was the constituents of the prototypical NF-κB complex, RelA and p50. However, the roles of RelB, c-Rel, and p52 are not yet firmly established. Initial exploration of these factors suggests that each can bind the HIV-1 promoter, but the effect of their binding is not clear (data not shown). Overexpression of RelB and c-Rel failed to induce appreciable expression of latent HIV-1 in the J-Lat system (data not shown), suggesting that these factors cannot direct the mechanisms employed by RelA to promote HIV-1 gene expression. Further studies to examine the ability of these factors to induce initiation and elongation of HIV-1 mRNA may be of interest. Additionally, analyses of chimeras formed with the DNA binding regions and transactivation domains of RelA, RelB, and c-Rel may identify previously unknown interacting partners that mediate the strong transactivating potential of RelA.

In our initial analysis of prostratin antagonism of HIV-1 latency, a potential role for NFAT emerged in experiments where PMA and ionomycin were used as agonists and cyclosporine A as an NFAT antagonist. The results suggest several basic questions. Can NFAT drive transcription of latent HIV-1 in the absence of NF-κB? If not, does NFAT

enhance initiation or elongation associated with NF-κB activation of latent HIV-1 transcription? Which NFAT family members mediate this effect? Is the effect direct? Can binding of NFAT family members be demonstrated at the latent HIV-1 LTR? What is the consequence of targeted knockdown of these factors? Our preliminary analyses of transcription factors bound to the latent HIV-1 LTR identified the constitutive binding of Sp1. In vitro studies of HIV transcription suggest that Sp1 is important in NF-κB-induced activation of HIV transcription. The contribution of Sp1 to HIV transcriptional regulation within the J-Lat system remains unexplored. Sp3 competes with Sp1 for the enhancer binding sites. It is unclear if Sp3 is bound to the HIV-1 LTR and, if so, whether a competitive equilibrium exists between these transcription factors. Additionally, it is unclear to what extent NF-κB will function in the absence of one or both of these factors. Further analysis of these basic transcription factors with demonstrated associations with the LTR should be fertile grounds for continued research.

Histone modification clearly plays an important role in the regulation of HIV-1 gene expression. Our studies demonstrate that histone H3 acetylation at LTR-proximal loci is associated with enhanced RNA polymerase II binding and increased transcriptional initiation. It is unclear to what extent other histone modifications are associated with transcriptional repression or activation. Studies of histone methylation at the latent LTR have not yet provided clear results; however, given its importance in general host gene regulation, this modification is likely an important regulator of HIV transcription. Additionally, the roles of histone phosphorylation, ubiquitylation, and ADP ribosylation in the context of the latent HIV-1 LTR have not been examined. Tools to manipulate these classes of histone modification do not yet exist, and consequently

while these modifications can be associated with transcriptional states, they can not be defined as causal factors. Future exploration of the role of histone modification will hinge on the development of new tools and technologies, allowing targeted intervention of these modifications.

A striking feature of the J-Lat system is the fractional activation of GFP expression induced by NF-κB agonists. Because the J-Lat model was selected clonally, a stimulated population would be predicted to respond to a given stimulus in an "all-or-none" manner. Instead, a reproducible fraction of cells is activated, with individual clones tending towards higher or lower percent response on a clone-by-clone basis. A potential explanation for this behavior is that the fraction of the clones not expressing HIV-1 in response to stimulation have lost the ability to express due to the accrual of random mutations in viral or host genes during propagation of the culture. However, when cells that failed to respond to an initial stimulus were sorted and subjected to a second round of stimulus, the fraction of cells expressing HIV-1 was similar to that obtained in the first round. Conversely, cells that had initially responded to stimulus only fractionally responded to a second stimulus. Taken together, these observation suggest that there is an inherently stochastic event that governs whether stimulus will induce gene expression or not in any particular latently infected cell. This phenomenon suggests that serial administration of antagonists of HIV latency will likely be required for effective purging of the reservoir.

p50-mediated recruitment of HDAC activity to the HIV LTR is a major contributor to transcriptional repression in the J-Lat system. Prior studies identified the interaction of the p50-homodimer with HDAC1, but these analyses stopped short of

identifying precise domains of either molecule required for this interaction. Determining which residues of p50 and HDAC1 are necessary to form this transcriptionally repressive complex would be useful for the design of agents to specifically block this interaction. Such a compound would likely have fewer side effects than generalized HDAC inhibition. Alternatively, displacing p50-p50 homodimers from κB enhancers would also alleviate transcriptional repression induced by this complex. The mechanism governing the fractional responsiveness of latent HIV-1 gene expression to NF-κB driven activation is not yet clear. Ectopic Tat expression strongly sensitizes J-Lat cells to NF-κB induced activation, changing the expression profile from a fractional response to a uniform response. The predominant model of Tat transactivation posits that Tat binds to initiated transcripts to enhance transcriptional activity. In this light, uniform expression of HIV-1 in the presence of ectopically expressed Tat in J-Lat cells suggests that activation of NF-κB induces universal transcriptional initiation. The failure of this universal initiation to translate into efficient gene expression in the majority of J-Lat cells in the absence of Tat suggests that a stochastic event lies downstream of transcriptional initiation. For example, the number of elongated transcripts in non-responding cells may be insufficient for the synthesis of Tat, precluding efficient elongation. Alternatively, induction of latent HIV gene expression may induce competing repressive processes that actively shut down HIV transcription. Further research into this basic question of mechanisms regulating the fraction responsiveness of J-Lat cells to NF-κB induction will undoubtedly be of interest.

While the J-Lat system replicates many aspects of HIV-1 latency in vivo, several factors set it apart from the scenario in infected patients. J-Lat cells replicate continuously and are highly metabolically active, whereas the key population of cells

within infected patients is largely quiescent. Our studies indicate that NF-κB can be induced as readily within quiescent cells as in J-Lat cells. Additionally, while the J-Lat model consists of several clonal populations, they may behave differently than the highly heterogeneous population in infected patients. Although the generality of a mechanism can be argued by demonstrating of an effect in several J-Lat clones, it is difficult to extend these arguments into in vivo systems given the strong selection for NF-κB-dependent expression inherent in the J-Lat system. Therefore, efforts to generate a model of heterogeneously integrated latent HIV-1 in primary lymphocytes would be of great value to future HIV-1 latency research.

Ultimately, advancement of our understanding of HIV-1 latency will require major improvements in our ability to analyze the latently infected population as it exists in the infected patient. Our studies of primary tissue were largely unsuccessful. We could only culture virus from a single patient (data not shown), and then could not propagate virus from this patient in subsequent studies. Our assay system depended upon viral propagation and identification of viral outgrowth by p24 ELISA. These assays might be improved with the use of more sensitive RT-PCR-based approaches for the quantitation of viral RNA. With sufficient assay sensitivity, viral gene expression could be assessed at both the initiation and elongation phases, allowing analysis of a broad range of potential antagonists of latency. Recently, a significant improvement in detection of latent virus from patient blood was made with leukophoresis, a process that increases the number of cells that can be isolated from patient blood [24]. The increased scale of lymphocyte recovery allowed consistent isolation of latent virus in infected patients and was sufficient for gross quantitation of the size of the latent pool.

Analyses of latent HIV-1 should not be restricted to peripheral blood lymphocytes (PBLs). Existing estimates of latent pool size are based entirely off of studies of PBLs, a tissue which may not accurately reflect the entire body load of latent HIV-1. Studies of lymph nodes, gut-associated lymphoid tissue (GALT), spleen, thymus, and other lymphocyte-rich environments may reveal disparate rates of latent HIV-1 infection. The concept of HIV-1 latency should be further broadened to encompass not only lymphocytes, but the entire range of HIV-susceptible cells, with particular attention to macrophages and dendritic cells, subsets of which may be very long-lived. To this end, the THP-1 macrophage model of latency and should be examined for similarities of mechanistic control with the J-Lat system [30].

In summary, these studies have opened a new chapter in the regulation of HIV transcriptional activity by the NF-κB family of transcription factors. Through a series of investigations, we have demonstrated the central importance of NF-κB in the activation of latent HIV-1 by the potential therapeutic prostratin. We described an unexpected role for NF-κB as a transcriptional repressor that reinforces HIV-1 latency through p50 recruitment of HDACs and chromatin-based restriction of RNA polymerase II accessibility. Finally, we uncovered a surprisingly complex kinetic profile of NF-κB induction of HIV transcription. These studies have opened the door for further investigation into the mechanistic control of transcriptional elongation by RelA. By elucidating mechanisms of transcriptional regulation of latent HIV-1, these studies

predict that in combination, NF-κB agonists, HDAC inhibitors and HIV-1 Tat should serve as a powerful activator of HIV-1 latency in HIV-1 infected patients.

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Appendix I

Host factors regulating postintegration latency of HIV

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Research Focus

Host factors regulating post-integration latency of HIV

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A recent study has provided important clues towards the identity of the host genes that conspire to promote post-integration latency of human immunodeficiency virus (HIV). Various genes controlling transcription, histone deacetylation and proteasome-mediated protein degradation have emerged as potential players. If the desired, but difficult, goal of complete virus eradication in HIV-infected patients is ever to be realized, the latent reservoir of HIV proviruses must be cleared. Understanding the molecular basis for viral latency is the key first step.

Introduction

Despite the notable success of combined antiviral therapy in reducing viral loads during infection with human immunodeficiency virus (HIV) and slowing clinical progression to acquired immunodeficiency syndrome (AIDS), the long-term use of these antiviral drugs has failed to achieve curative viral eradication in HIV-infected patients. This failure is due, at least in part, to a pool of residual memory CD4 T cells containing integrated but transcriptionally dormant HIV proviruses (Figure 1). Although small in number (10^5–10^6 per patient), this latent reservoir is established within days of the initial infection [1] and is quite long-lived (the estimated half-life of memory CD4 T cells is at least 44 months) [2]. Owing to this long half-life, elimination of the latent reservoir is estimated to require the administration of antiviral drugs for 60+ years (Box 1). Clearly, a different approach to the problem posed by latent proviruses is needed.

Hope springs from the fact that latent proviruses can be awakened from their transcriptional slumber by stimulating the host T cells with mitogens [3] or anti-CD3-CD28 antibodies [4]. Unfortunately, in clinical trials, OKT3 anti-CD3 antibodies and interleukin (IL)-2 have failed thus far to eliminate the latent pool, even though the size of the reservoir was reduced [5,6]. Translation of these findings into practical therapies will require a deeper understanding of how latency is established in the first place. Three general mechanisms have been considered to date: (i) a repressive chromatin environment, (ii) the lack of action and/or accessibility of the HIV long terminal repeat (LTR) to key host transcription factors, and (iii) a failure of Tat, the transcriptional activator of HIV, to act. Rather than reflecting a single mechanism (Box 2), HIV latency might involve a combination of these different mechanisms, posing an even more daunting therapeutic challenge.

The recent identification of changes in cellular gene expression in latently infected cells by Krishnan and Zeichner [7] confirms and extends the mechanistic possibilities. These investigators detected changes in host gene expression, including specific transcription factors, histone deacetylases and proteasome genes, which might play key roles in the establishment and maintenance of HIV latency.

Viral defects promoting viral latency

Early studies of HIV latency were propelled by the identification of chronically infected transformed cell lines, in which proviral gene expression depends upon cellular activation. Subsequent investigations of these cell lines revealed mutations in the viral transcriptional transactivating gene *Tat* [8], or in the Tat-responsive RNA element located at the 5′ end of all viral mRNAs [9]. Although these results underscore the ability of select mutations in the viral genome to promote latency, these

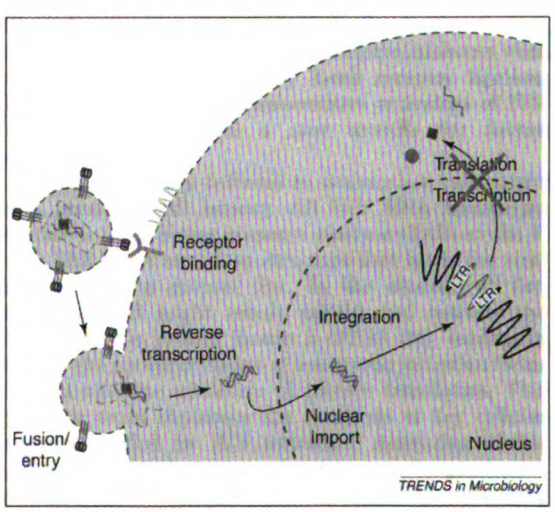

Figure 1. The molecular basis of HIV Latency. In the normal life cycle of HIV, virion particles (i) fuse with target cells, enabling insertion of the viral core containing the viral RNA genome. This step is followed by reverse transcription of the genomic viral RNA (ii) producing a double stranded DNA viral genome, which is (iii) imported into the nucleus, and (iv) integrated into the host genome. Following establishment of the HIV provirus in the nucleus, (v) viral gene expression usually commences, which involves the action of various host transcription factors. In the case of latent HIV infection, viral gene expression does not occur until the cellular host becomes activated.

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Box 1. Characteristics of latently infected memory T cells

- Estimated size: unknown (estimated to be 10^5–10^6 per patient).
- Half-life: >40 months.
- Time to elimination with highly active anti-retroviral therapy (HAART): >60 years.

changes do not reflect events underlying HIV latency *in vivo* because replication-competent rather than defective viruses are induced from the latent reservoir after cellular activation. However, it is notable that both HIV Tat and the host NF-κB transcription factor, which are potent activators of the HIV LTR, use a similar strategy to stimulate the expression of the HIV provirus [10]. Both engage the p-TEFb complex (consisting of cyclin T1 and CDK9), which phosphorylates the C-terminal domain of previously bound RNA polymerase II complexes. This multi-site phosphorylation event converts the polymerase from a weakly processive to a highly processive enzyme capable of copying the entire viral DNA template into RNA. In the absence of NF-κB or Tat, the bound polymerase moves only a few hundred bases down the template before stalling and disengaging [11]. These effete polymerase complexes thus generate only short, non-functional viral transcripts.

A key unanswered question is whether latently infected cells produce such short viral transcripts. Recent efforts aimed at analyzing HIV transcripts in bulk cultures before the addition of activating signals have revealed the presence of short viral transcripts [12]. However, the fact that integrated but defective viral genomes are much more common than latent genomes complicates the interpretation of these results because the short transcripts could be derived from defective integrated viruses rather than latent replication-competent proviruses.

Cellular factors controlling viral latency

A relative deficiency of key stimulatory transcription factors or co-activators might also underlie HIV latency. Quiescent resting memory CD4 T lymphocytes harboring latent provirus maintain such a strikingly low level of transcription that the necessary factors for HIV provirus expression might be limiting [13]. Uncertainty also surrounds how infection of these resting memory CD4 T cells is established in the first place. One possibility is that infection occurs as the memory CD4 T cell undergoes the transition from an active to a resting state. During this narrow temporal window, the T cells might be sufficiently activated to support reverse transcription, nuclear translocation and viral integration, but later steps in the viral life cycle are short-circuited by inadequate cellular activation. Such a model could explain why infected patients harbor so few latently infected cells. Recent efforts to induce expression of latent proviruses have focused on activation of transcription factors by a range of immunostimulatory agents, including IL-2, IL-7 and the non-tumor-promoting phorbol ester, prostratin [14,15].

Box 2. Mechanisms generating HIV latency

- Viral genome integration into repressive heterochromatin environment.
- Lack of host transcriptional activators.
- Lack of, or dysfunctional, Tat.
- Accelerated proteasome-mediated degradation of key viral or host regulatory proteins.
- Innate host antiviral processes (PKR/siRNA).

Although HIV integration preferentially occurs in actively transcribed host genes [16], some proviruses integrate into heterochromatic regions of the genome where transcription is normally repressed [17]. Thus, chromatin structure surrounding the locus of HIV provirus integration could play an important role in determining whether a particular provirus enters latency or not. Krishnan and Zeichner [7] have detected upregulation of two histone deacetylases (HDAC1 and HDAC2) in latently infected cells. These enzymes promote the deacetylation of histone tails – a change associated with transcriptional repression. They also observed increased expression of the YY1 transcription factor, a known recruiting factor of histone deacetylases to the HIV LTR [7]. As such, upregulation of YY1 could promote transcriptional silencing of the integrated provirus [18]. These results suggest that cells expressing genes that promote histone deacetylation, and an overall more repressive chromatin environment, could play an important role in the generation of latent HIV proviruses. In support of this notion, HIV transcription in latently infected cell lines is often activated by the addition of the HDAC inhibitor, trichostatin A [19].

Krishnan and Zeichner [7] also reported increased expression of various proteasome subunit genes. These findings raise the possibility that key factors required to effectively activate the HIV LTR might be subject to accelerated degradation by the proteasome. In support of this possibility, the addition of proteasome inhibitors successfully activated latent HIV proviruses in those cell lines that were analyzed. Indeed, the levels of virus activation achieved with these proteasome inhibitors were similar to that produced by T-cell receptor ligation. Identification of proteasome-sensitive activators of HIV transcription provides a new avenue for future investigation.

Natural antiviral defenses in mammalian cells might also promote viral latency. All HIV RNA transcripts contain a *trans*-acting response element (TAR) at the 5′ end. TAR has a stem-loop structure that binds the viral transactivating protein Tat. In the absence of Tat, however, TAR might mimic dsRNA and activate the RNA-inducible protein kinase R (PKR) [20], leading in turn to the phosphorylation of eukaryotic initiation factor (eIF)2α and the inhibition of protein translation. This pathway could shutdown the synthesis of key cellular proteins needed for HIV activation. Activation of this pathway would of course be dependent on the generation of viral RNA. It is possible that the short TAR-containing viral RNAs generated by the bound, but poorly processive, RNA polymerase II complex would be sufficient. Another entirely untested but intriguing possibility is that HIV dsRNA might serve as a substrate for the Dicer complex and lead to the generation of HIV-specific small interfering (si)RNAs that promote latency by degrading HIV transcripts and silencing transcription.

HIV latency *in vivo* remains a 'black box'

Among the challenges posed by HIV latency is the rarity of latently infected cells in patients. In addition, these cells lack any identifying surface markers that would facilitate their separation from normal uninfected cells. Consequently, latently infected cells cannot be specifically purified for study. Instead, scientists are left with stimulating bulk populations of resting memory CD4 T cells in which only a small fraction of the cells contain latent provirus. This approach was used successfully to demonstrate the existence of an inducible latent reservoir within memory CD4 T cells and to estimate the relative frequency of these cells *in vivo* [21]. However, the indirect nature of this experimental approach does not allow for dissection of the underlying molecular basis for HIV latency. Accordingly, the site of proviral integration, the extent of viral RNA synthesis, expression of HDACs, chromatin environment, level of proteasome activity, and the relative abundance of key transcription factors within the latently infected cell *in vivo* remain undefined.

Concluding remarks

Latent infection of memory CD4 T cells continues to pose a major obstacle to the successful eradication of HIV in infected patients. Latent proviruses within other currently undefined cellular reservoirs could also exist. It remains unknown whether HIV latency is derived from a single mechanism or reflects a composite of different mechanisms. If multiple mechanisms are involved, finding effective methods to completely purge the virus will be even more complex. A key for future progress is the establishment of cell-culture systems that faithfully model HIV latency *in vivo*. Only by using such cells will new insights into the molecular basis for latency emerge. However, it will be important to validate these proposed mechanisms in latently infected cells isolated from patients. The recent description of genes differentially expressed in latently infected cell lines provides new and interesting leads that must now be tested in primary cells. Only by understanding the molecular underpinnings of HIV latency can we hope to develop effective strategies to eliminate this reservoir, thereby realizing the vaunted but difficult goal of complete HIV eradication.

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