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T-cell receptor signaling enhances transcriptional elongation from latent HIV proviruses by activating P-TEFb through an ERK-dependent pathway

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Abstract

Latent HIV proviruses are thought to be primarily reactivated *in vivo* through stimulation of the T-cell receptor (TCR). Activation of the T-cell receptor (TCR) induces multiple signal transduction pathways leading to the ordered nuclear migration of the HIV transcription initiation factors NF- κ B and NFAT as well as potential effects on HIV transcriptional elongation. We have monitored the kinetics of proviral reactivation using chromatin immunoprecipitation (ChIP) assays to measure changes in the distribution of RNA polymerase II (RNAP II) on the HIV provirus. Surprisingly, in contrast to TNF- α activation, where early transcription elongation is highly restricted due to rate-limiting concentrations of Tat, efficient and sustained HIV elongation and P-TEFb recruitment is detected immediately after activation of latent proviruses through the T-cell receptor. Inhibition of NFAT activation by cyclosporine had no effect on either HIV transcription initiation or elongation. However, examination of P-TEFb complexes by gel filtration chromatography showed that TCR signaling led to the rapid dissociation of the large inactive 7SK RNP complex and release of active low molecular weight P-TEFb complexes. Both P-TEFb recruitment to the HIV LTR and enhanced HIV processivity were blocked by the ERK kinase inhibitor U0126 but not by AKT and PI3 kinase inhibitors. In contrast to treatment by HMBA and DRB, which disrupt the large 7SK RNP complex but do not stimulate early HIV elongation, TCR signaling provides the first example of a physiological pathway that can shift the balance between the inactive and active P-TEFb pools and thereby stimulate proviral reactivation.

Keywords

HIV latency; HIV transcription; P-TEFb; T-cell receptor signaling; ERK signaling

Introduction

HIV is able to evade antiviral immune responses and antiretroviral therapy by establishing latent infections, most notably, in the long-lived resting memory CD4⁺ T-cell population

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(for reviews, see^{1; 2; 3}). These latent infections are the major obstacle to virus clearance and the virus will rapidly rebound from the latent reservoir following the interruption of antiretroviral therapy even in patients undergoing HAART where plasma viremia has been undetectable for many years^{4; 5; 6}. Unfortunately, intensification of antiretroviral therapy does not appreciably deplete the residual viremia of patients on therapy⁷. The failure of current therapy to eradicate HIV has prompted renewed efforts to define the molecular mechanisms leading to HIV latency and to develop new therapeutic tools to attack the latently infected cells^{8; 9; 10}.

HIV transcription is dependent upon the expression of the viral trans-activator protein, Tat which fuels a powerful feedback mechanism (for reviews, see^{11; 12; 13}). In the absence of Tat transcription initiation is normal but elongation is highly restricted and only short abortive transcripts are produced. Tat acts to stimulate transcription elongation by recruiting the cellular transcriptional elongation factor P-TEFb¹⁴ to nascent RNA polymerases that have transcribed through the HIV TAR element, an RNA stem-loop structure found at the 5' end of all viral transcripts. The form of P-TEFb used to stimulate HIV transcription is a complex of human cyclin T1 (hCycT1), which is an RNA-binding protein that binds cooperatively to TAR RNA together with Tat and CDK9, a protein kinase that phosphorylates a variety of proteins within the elongating transcription complex. These phosphorylation events result in both the removal of the block to elongation through the targeting of the E (RD) subunit of the elongation repressive factor NELF¹⁵ and positive events that include the phosphorylation of the C-terminal domain (CTD) of RNAP II¹⁶ and Spt5, a subunit of the DRB-sensitivity inducing factor (DSIF) that enhances transcriptional elongation^{17; 18}.

Because Tat functions as part of a positive regulatory circuit, conditions that restrict transcription initiation will in turn cause a reduction in Tat levels to below threshold levels and lead to the establishment of latency^{19; 20}. Typically, epigenetic silencing of HIV transcription initiation provides the trigger that drives viruses into latency. Key silencing events include the establishment of heterochromatic structures through the recruitment of histone deacetylases (HDACs)^{21; 22; 23}, induction of histone methylation^{24; 25; 26; 27}, and DNA methylation^{28; 29}. In addition there have been documented examples of HIV silencing through promoter occlusion when the viruses have integrated into actively transcribed genes^{30; 31; 32}. However, even in these instances establishment of heterochromatic structures on the latent provirus appears to contribute to silencing of HIV transcription. These epigenetic blocks can be effectively reversed by the transcriptional initiation factors NF- κ B and NFAT^{24; 25; 33; 34}. Both quiescent T-cells and Jurkat T-cells restrict HIV transcription initiation by sequestering the cellular transcription initiation factors NF- κ B and NFAT in the cytoplasm^{35; 36}.

In actively replicating cells, such as HeLa cells and Jurkat T-cells, P-TEFb activity is tightly regulated and the majority of the enzyme is sequestered into a large inactive 7SK RNP complex comprising 7SK RNA and a series of RNA binding proteins^{37; 38}. Essential components of the 7SK RNP complex include HEXIM1 or HEXIM2, which inhibit the CDK9 kinase in a 7SK-dependent manner^{39; 40}, LARP-7, a La-related protein bound to the 3' UUU-OH sequence of 7SK^{41; 42}, and BCDIN3, a methylphosphate capping enzyme specific for 7SK⁴³. This restriction of enzymatically active P-TEFb in the cell provides an additional block to efficient transcription elongation from the HIV promoter. Tat overcomes this barrier by disrupting the 7SK RNP complex by competitively displacing HEXIM-1^{44; 45; 46}.

Resting CD4⁺ T-cells further ensure that latent proviruses remain transcriptionally inactive by restricting both the levels of CycT1⁴⁷ and the levels of phosphorylated CDK9, which is

the enzymatically active form of the enzyme^{47; 48}. Similarly, in unstimulated monocytes translation of CycT1 is blocked miR-198⁴⁹.

Recently we have developed model systems for studying HIV latency using lentiviral vectors that express attenuated Tat genes *in cis*^{24; 25}. Detailed kinetic studies have emphasized that proviral reactivation following NF- κ B mobilization by TNF- α results in sequential waves of RNAP II recruitment to the LTR as NF- κ B enters and exits the nucleus, but virtually no downstream RNAP II until Tat is synthesized 2 to 4 hr after stimulation^{25; 33; 50}. In order to define the mechanisms of proviral activation following TCR activation we performed a similar kinetic analysis of proviral induction. We found unexpectedly that TCR signaling results in the immediate activation of transcription elongation, even at times when Tat levels are too low to sustain transcription elongation. This early increase in elongation is due to the activation of P-TEFb by the disruption of the 7SK RNP complex through the ERK pathway.

Results

Induction of latent provirus by T-cell receptor activation

The 2D10 cell line is a latently infected Jurkat T-cell line carrying a lentiviral vector that expresses the regulatory proteins Tat and Rev *in cis* and a short-lived green fluorescent protein (d2EGFP) in place of Nef²⁵. The provirus in 2D10 cells also carries the H13L mutation in Tat which effectively supports HIV transcription elongation but is attenuated and therefore helps to promote proviral entry into latency^{24; 25; 51}. Extensive characterization of the 2D10 cell line has shown that the provirus is inserted into the first exon of the SEPX1 gene in the opposite orientation from the SEPX1 promoter²⁵.

An important advantage of 2D10 cells is that although HIV gene expression is highly suppressed (more than 98% of the uninduced cells are d2EGFP negative) the cells are readily induced. For example, over 99% of the cell population becomes d2EGFP positive ($\text{mfi} > 2 \times 10^1$) after exposure to 10 ng/ml TNF- α for 18 hr (Figure 1A). The provirus in 2D10 cells can also be efficiently reactivated by stimulation of the T-cell receptor using a combination of α -CD3 mAb and α -CD28 mAb. As shown in Figure 1A mAb stimulation of the TCR resulted in 62.4% reactivation of the latent proviruses (Figure 1A).

In addition to activating NF- κ B, TCR stimulation induces a complex cascade of pathways, including activation of NFAT through the Ca²⁺-calcineurin pathway. This raises the intriguing possibility that HIV proviral reactivation following TCR stimulation involves multiple transcription factors in addition to NF- κ B. In order to monitor the kinetics of activation of transcription factors following TCR stimulation we compared the nuclear levels of NF- κ B (p65, p50) and NFAT (NFATc1 and NFATc2) following either TNF- α treatment or stimulation of the TCR with α -CD3/ α -CD28 mAb by Western blotting (Figure 1B). During a 6 hr time course TNF- α induced cycling of the NF- κ B p65 and p50 subunits between the cytoplasm and nucleus^{25; 33; 50}. After an initial peak of nuclear accumulation at 30 min, both NF- κ B p65 and p50 levels oscillated with a period of approximately 2.5 hr. As expected, there was no detectable increase in the nuclear levels of either NFATc1 or NFATc2 following TNF- α treatment. NFATc2 was undetectable in the nucleus prior to TNF- α exposure, whereas NFATc1 showed moderate nuclear levels in unstimulated cells which slowly fell during the 8 hr time course.

Stimulation of the TCR with α -CD3/ α -CD28 mAbs activated both NF- κ B and NFAT with unique kinetics (Figure 1B). Initially, entry of NF- κ B p65 into the nucleus followed kinetics that were nearly identical to those observed following TNF- α stimulation and nuclear p65 levels reached a maximum at 30 min. However, in contrast to activation by TNF- α , p65

levels then declined rapidly and showed only slight peaks of activation at 2.5 and 5.5 hr. NF- κ B p50 also showed a distinct initial peak at 30 min followed by more sustained activation during the next 8 hr. NFATc2 reached maximal nuclear levels at 30 min before declining to basal levels by 2.5 hr, whereas NFATc1 levels gradually increased before reaching maximal levels between 2.5 and 8 hr after TCR stimulation.

NFAT is not required for HIV transcription in Jurkat T-cells following TCR activation

Since NFATc1 is induced in response to TCR signaling, it is possible that it contributes to HIV transcription under these conditions. To test this possibility, we used cyclosporine A (CsA) to block the Ca^{2+} -calcineurin activation pathway. Control experiments demonstrated that CsA was able to completely block the induction of both NFATc1 and NFATc2 but did not prevent NF- κ B p65 or p50 induction (data not shown). Flow cytometry (Figure 1A) demonstrated that CsA did not block proviral reactivation following either TNF- α , or TCR stimulation, suggesting that not only is NFAT dispensable for proviral reactivation, but sufficient levels of NF- κ B p65 were induced under both conditions to drive HIV transcription.

Kinetics of HIV transcription in response to TCR signaling

In order to study early events during the induction of transcription from the latent HIV proviruses we performed quantitative ChIP assays using an RNAP II antibody (Figure 2). The ChIP assays were used to measure the accumulation of RNAP II at the TAR region near the promoter (+30 to +134), which provides an accurate estimate of transcription initiation rates. In addition, RNAP II levels at a downstream region (+2593 to +2691) were measured to provide an assessment of transcription elongation efficiencies.

The data shown in Figure 2 is a subset of an extensive data set used to simultaneously monitor HIV transcription and gene expression from control cellular genes that are responsive to NF- κ B and NFAT. The ChIP data were analyzed by fitting a series of Gaussian distributions. This procedure effectively allows for the objective identification of the main peaks of transcription and the relative contribution of successive waves of transcription factor activation to overall transcription. The full data set is provided as supplementary material along with details of the curve fitting.

As we have previously reported, a low level of RNAP II transcription complexes accumulate near the start site of transcription of latent HIV proviruses^{25; 50; 51}. Both TNF- α stimulation and α -CD3/ α -CD28 mAb co-stimulation led to the rapid recruitment of RNAP II to the HIV promoter concomitantly with the initial rise of NF- κ B levels in the nucleus. Under both stimulation conditions, RNAP II accumulation at the promoter reached maximal levels at 30 min and was nearly identical during the first 90 min.

Following the initial cycle of transcription initiation, the kinetics of HIV proviral reactivation after TNF- α treatment and TCR stimulation diverged significantly. In the case of TNF- α activation, transcription initiation rebounded by 3.5 hr and increased throughout the subsequent cycles of NF- κ B p65 entry into the nucleus during the next 8 hr. By contrast, following TCR activation, maximal HIV transcription initiation was achieved during the first cycle and lower levels of transcription initiation were observed during each of the later time points.

The I κ B α gene, which is highly dependent on NF- κ B for transcription initiation, but does not require Tat for transcription elongation, was analyzed in parallel to the HIV provirus in order to provide an internal control for NF- κ B activity (Figure 2B). Accumulation of RNAP II near the promoter of the I κ B α gene (+155 to +237) was used as a measurement of transcription initiation, while accumulation of RNAP II in a downstream region (+2038 to

+2159) was used to evaluate transcription elongation. As in the case of the HIV provirus, there was a rapid increase in transcription initiation of the $\text{I}\kappa\text{B}\alpha$ gene during the first cycle of NF- κB p65 entry into the nucleus and the first peak of transcription initiation was nearly identical following either TNF- α activation or TCR stimulation. However, comparing the patterns of $\text{I}\kappa\text{B}\alpha$ gene transcription and HIV proviral transcription at later times demonstrates that the HIV promoter is both more strongly stimulated and sustains a higher level of transcription than the $\text{I}\kappa\text{B}\alpha$ gene between 2 and 8 hr. Similarly, following TCR activation, $\text{I}\kappa\text{B}\alpha$ gene responds poorly after the initial cycle of activation whereas there is substantial HIV transcription under these conditions. The simplest explanation for these kinetic differences is that NF- κB p65 is the primary factor driving $\text{I}\kappa\text{B}\alpha$ gene transcription throughout the time course, whereas in response to TCR signaling additional factors help to sustain HIV transcription following the initial phase of NF- κB p65 activation.

TCR activation stimulates HIV transcriptional elongation

Although the induction of HIV transcription initiation by TNF- α was very efficient during the first cycle of NF- κB p65 entry into the nucleus, downstream RNAP II levels remain at extremely low levels at this time and only began to rise after 2 hours of stimulation when Tat protein begins to accumulate^{25; 33}. In contrast, stimulation of 2D10 cells by $\alpha\text{-CD3}/\alpha\text{-CD28}$ antibodies strongly induced elongation during the first 90 min, a time prior to the synthesis of new Tat protein. Integration under the fitted curves demonstrated that although initiation during the first cycle of transcription is nearly identical when cells are stimulated either by TNF- α , or through the TCR, elongation is approximately twice as efficient following TCR activation.

Changes in elongation efficiency of the HIV provirus and the $\text{I}\kappa\text{B}\alpha$ gene following activation by TNF- α or TCR stimulation were estimated by taking the ratio of RNAP II at the promoter and the downstream region for the HIV provirus (Figure 2A) and the $\text{I}\kappa\text{B}\alpha$ gene (Figure 2B). Following stimulation by TNF- α , there is a progressive increase in elongation efficiency between 2 and 8 hr following an initial lag period of 2 hr. Although initiation rates oscillate throughout this time course the elongation ratios increase monotonically, suggesting that this is due to accumulation of Tat in the system. During TCR activation changes in the elongation efficiency are apparent at the earliest time points and maximal transcriptional elongation efficiencies are reached sooner than during TNF- α activation. Compared to the $\text{I}\kappa\text{B}\alpha$ gene (Figure 2B), the elongation efficiency of the latent HIV provirus is initially significantly more restricted (i.e. 10% versus 20% to 30%) but becomes dramatically more efficient (i.e. 75% compared to 30%) once Tat is induced. In contrast to the results observed with the HIV provirus, the elongation efficiency from the $\text{I}\kappa\text{B}\alpha$ gene remains constant during the activation time course since it is not activated by Tat.

TCR stimulation induces P-TEFb recruitment to the HIV provirus

In order to examine if the strong activation of elongation we observed at early times after TCR stimulation was associated with enhanced P-TEFb recruitment to the HIV provirus, we measured the levels of RNAP II and CDK9 at various regions of HIV provirus by ChIP assays. Figure 3A shows that although the amounts of RNAP II near the transcription start site are similar after stimulation for 30 min by either TNF- α , or through the TCR, the amount of RNAP II found downstream of the TAR sequence was significantly higher in the TCR activated samples. This enhanced RNAP II processivity was associated with a 3- to 6-fold increase in CDK9 levels in downstream regions of the provirus (Figure 3B). Since, as described above, there is no significant increase in newly synthesized Tat until at least 120 min following stimulation by TNF- α , these results imply that TCR-mediated signaling uniquely increased the availability of either P-TEFb or Tat.

Enhanced HIV transcriptional elongation is mediated through an ERK-dependent signaling pathway

To define the molecular mechanisms responsible for increasing P-TEFb pool sizes, 2D10 cells were treated with a range of activators and inhibitors of pathways known to be induced by TCR-mediated signaling (Figure 4). As shown in Figure 4A, activation of the TCR led to a rapid increase in the amount of phosphorylated ERK1/2 and phosphorylated AKT in both the nucleus and the cytoplasm. Treatment of cells with U0126, an inhibitor of the kinase activity of MAP kinase kinase (MAPKK), selectively blocked ERK1/2 phosphorylation but did not affect AKT phosphorylation. Conversely, treatment of cells with LY294002, a selective phosphatidylinositol 3-kinase (PI3K) inhibitor, blocked AKT phosphorylation, and increased ERK1/2 phosphorylation, since blocking of the AKT pathway removes an inhibitor of the ERK pathway^{52; 53}. For example, in the experiment shown in Figure 4A, at 15 min after TCR stimulation phospho-ERK-2 levels were 2.9-fold higher in cells treated with LY294002 than in control cells and phospho-ERK-1 levels were 1.7-fold higher than in control cells. Treating cells with a combination of LY294002 and U0126 reduced ERK2 levels to 60% of the control values.

ChIP assays were performed to study the effects of these inhibitors and a range of transcription activation conditions on transcription elongation. In the experiments shown in Figure 4B, RNAP II levels were measured at both an upstream (+30 to +134) and a downstream (+2593 to +2691) site and the ratio used to estimate the elongation efficiency, as described above. Exposure of 2D10 cells to TNF- α , PHA, TSA or HMBA did not cause any significant changes in elongation efficiency at 30 min following treatment. However, activation by PMA and a combination of PMA and PHA strongly induced elongation at 30 min post stimulation.

In cells activated either through the TCR or by PMA, exposure to U0126 reduced elongation efficiency by approximately 50%, but had no significant effect on initiation (Figure 4B). By contrast, the AKT inhibitors LY294002 and AI8 (Akt inhibitor 8) were able to further activate transcriptional elongation following TCR stimulation or PMA activation. Since U0126 is able to partially reverse the effects of LY294002, it seems likely that the enhancement in elongation that we have observed is due to the enhanced activation of ERK-1/2 that is mediated by AKT inhibition.

Control experiments confirmed that none of the inhibitors studied affected transcriptional elongation in untreated cells or in cells activated by TNF- α (Figure 4B). Finally, we note that blocking of NFAT activation by CsA had no impact on transcription elongation (Figure 4B).

CycT1 and CDK9 levels are not altered in response to TCR signaling

The signaling events stimulated by the TCR could either result in enhanced synthesis of components of the transcription elongation machinery, such as CycT1 and CDK9, or act to enhance their activity by inducing post-transcriptional modifications. To distinguish between these two possibilities additional Western blotting experiments were undertaken to test whether nuclear ERK, CycT1 and CDK9 levels were altered within the first 2 hr after TCR activation. As shown in Figure 5A, nuclear ERK-1 levels remain constant during the first hr after TCR activation. By contrast, high levels of phosphorylated ERK-1/2 appear in the nucleus within 15 min and then slowly decay. Consistent with the results shown in Figure 4A, the phosphorylation of ERK-1/2 is blocked by U0126.

There are no detectable changes in the nuclear levels of CycT1, CDK9 or CDK7 during the first 2 hr after TCR stimulation (Figure 5B). The constitutive expression of CycT1 and CDK9 in Jurkat T cells is in contrast to the situation in primary resting memory T-cells and

monocytes where CycT1 are low prior to TCR stimulation and new CycT1 is synthesized within 60 min^{24; 48; 49}.

TCR signaling induces global increases of P-TEFb association with chromatin

In order to test whether TCR signaling induced a general increase in transcription associated with P-TEFb recruitment to the chromatin, we performed Western blots on a series of nuclear extracts (Figure 5C). In the Jurkat T-cells there is an excess of HEXIM1 and CDK9 which can be detected at high levels in cytoplasmic extracts prepared in hypotonic buffers. CycT1 is absent from these fractions. By contrast, nuclear extracts prepared using buffers containing 0.36 M NaCl, contain stoichiometric amounts of CycT1 and CDK9 and variable amounts of HEXIM1. The initial nuclear extracts contain high levels of HEXIM1 suggesting that they are enriched in the nucleoplasmic 7SK RNP complex. Subsequent extracts, which represent fractions that are more tightly associated with the chromatin, contain only CycT1 and CDK9. Treatment of cells with either DRB or actinomycin D (Act D), which induce dissociation the 7SK RNP complex^{37; 38} (see also Figure 8 below) induced increased association of the CycT1 and CDK9 subunits of P-TEFb with the chromatin, but did not alter HEXIM1 levels in the nuclear extracts. Similarly, TCR signaling induced a global increase in P-TEFb association with the chromatin. This is consistent with the ChIP data presented in Figure 3 demonstrating that TCR signaling increased the levels of CDK9 recruited to latent HIV proviruses.

Thus, the activation of elongation on HIV by TCR signaling that we have observed seems to be associated with an ERK-mediated post-translation modification of presynthesized P-TEFb rather as the result of new synthesis of its subunits.

U0126 blocks TCR activation of HIV transcriptional elongation

To verify that elongation is selectively activated early after TCR activation, we performed time course experiments similar to those shown in Figure 2 in the presence and absence of U0126. U0126 selectively blocked ERK phosphorylation, but it did not interfere with NF- κ B p65 and p50 translocation to the nucleus following TCR (Figure 6A). U0126 also had no impact on NFATc1 and NFATc2 activation. In the corresponding ChIP experiments, U0126 treatment did not inhibit RNAP II recruitment to the promoter but did result in a dramatic decrease in HIV elongation during the first wave of transcription (Figure 6B). Similarly, U0126 is able to restrict transcriptional elongation and CDK9 recruitment following PMA stimulation of cells (data not shown). U0126 also strongly inhibits HIV elongation during the 2 to 4 hr period when Tat synthesis has resumed. Complementary studies using PD98059, which inhibits the dephosphorylated form of mitogen-activated protein kinase kinase-1 (MAPKK1) and blocks the induction of AP-1 during TCR signaling have shown that AP-1 contributes to HIV transcription during the 2 to 4 hr period but plays no role during the first round of HIV elongation between 0 to 2 hr (Hokello, J., Mbonye, U., & Karn, J., unpublished).

Recruitment of P-TEFb to HIV proviruses following PMA activation is blocked by U0126 and enhanced by LY294002

The inhibitor studies shown in Figure 4 strongly suggested that both TCR activation and PMA stimulation enhanced HIV transcription elongation through a common mechanism involving the ERK kinase pathway. To confirm that PMA stimulation of cells resulted in enhanced P-TEFb recruitment to the HIV provirus additional ChIP experiments were performed (Figure 7).

Activation of cells for 30 min with PMA stimulated both RNAP II recruitment to the provirus, transcriptional elongation, and CDK9 recruitment. Both recruitment of RNAP II

and CDK9 were strongly inhibited by treatment with U0126. Importantly, treatment of cells with a combination of PMA and LY294002 boosted the levels of both RNAP II and CDK9 associated with the activated HIV provirus and was inhibited by U0126. Thus the amount of RNAP II and CDK9 associated with the HIV provirus varied in proportion to the degree of ERK activation.

Disruption of the 7SK RNP complex is mediated by ERK

The preceding data strongly suggests that restrictions imposed on P-TEFb activity in resting T cells can be relieved through activation of the MAPKK/ERK pathway. We therefore decided to investigate whether TCR activation could alter the levels of the 7SK RNP complex found in T-cells.

As shown in Figure 8A, fractionation of Jurkat T-cell extracts by gel filtration chromatography demonstrates that the majority of P-TEFb is found as part of the large 7SK RNP complex. Integration of the areas under the peaks showed that in the unstimulated cells 95% of the CDK9 and 68% of the HEXIM-1 in the cell is associated with the large complex while 5% of the CDK9 and 32% of the HEXIM-1 were associated with the low molecular weight fraction. Thus, there is a significantly higher proportion of CDK9 associated with the 7SK RNP complex in Jurkat T-cells than has been observed in the more extensively studied HeLa cells where typically less than 50% of the P-TEFb in the cell is in the large complex^{37; 38; 46; 54}.

As shown in Figure 8A, stimulation of the cells for 30 min through the TCR or by exposure to PMA results in a significant disruption of the large complex and release of P-TEFb into a low molecular weight fraction. Following TCR treatment 29% of the CDK9 and 35% of the HEXIM-1 is found in the lower molecular weight fractions. The degree of complex dissociation following TCR signaling is comparable to the disruption seen following 30 min exposure to HMBA (low molecular weight fraction: CDK9, 26%; HEXIM1, 47%), but less than that induced by DRB (low molecular weight fraction: CDK9, 60%; HEXIM1, 88%) (Figure 8B).

U0126 effectively blocks the disruption of the 7SK RNP complex by TCR signaling. In the experiment shown in Figure 9A, 12% of the CDK9 and 26% of the HEXIM-1 was found initially in the low molecular weight fraction. After stimulation of the TCR for 30 min, 22% of the CDK9 and 31% of the HEXIM1 was found in the low molecular fraction. Treatment with U0126 not only blocked TCR induced dissociation but resulted in slightly more accumulation of CDK9 and HEXIM1 in the 7SK RNA/P-TEFb/HEXIM1 complex than in unstimulated cells (93% CDK9 and 88% HEXIM1 in the complex). This strongly suggests that the ERK signaling is responsible for the release of functional P-TEFb that we have described above.

Although activation of cells through the TCR or by PMA and treatment with HMBA and DRB all resulted in 7SK RNP disruption, only activation by TCR or PMA resulted in enhanced HIV elongation efficiencies. Since DRB is also an inhibitor of CDK9 activity, it would be expected to only release inactive P-TEFb.

The failure of HMBA to stimulate HIV transcription elongation appears to be associated with the phosphorylation state of CDK9. Enzymatically active CDK9 must be phosphorylated on Thr186. As shown in Figure 9B, disruption of the 7SK RNP complex by TCR signaling resulted in the release of CDK9 that remains phosphorylated on Thr186. In this experiment 95% of the CDK9 in the unstimulated cells remained associated with the large complex and 5% was found in the small complex. Similarly, the large complex contained 92% of the phospho-Thr-CDK9 while the small complex contained 8%. After

TCR stimulation, 21% of the total CDK9 and 11% of the phospho-Thr-CDK9 was released into the small complex. However, as originally reported by Chen et al.⁵⁴, although HMBA induced release of 45% of the CDK9 into the small complex, the released P-TEFb was extensively dephosphorylated on Thr-186. For example, the amount of phospho-Thr-CDK9 in the small complex following HMBA treatment corresponded to only 18% of the total CDK9.

TCR-mediated disruption of the 7SK RNP complex does not require Tat

Previous studies have documented that Tat acts to disrupt the 7SK RNP complex by competing with HEXIM for CycT1 binding^{44; 45; 46}. In order to determine whether residual Tat present in the latently infected 2D10 cells could be contributing to the disruption of the 7SK RNP complex we measured the dissociation of the 7SK RNP complex in response to TCR signaling and PMA treatment in 2B2D cells²⁵, which harbor a latent provirus carrying the inactive C22G mutation in Tat, which prevents its interactions with P-TEFb (Figure 10). As shown in Figure 10A, both TCR and PMA are able to induce 7SK RNP complex disruption in 2B2D cells, at levels comparable to those seen in the 2D10 cells. For example, in the uninduced cells 86.5% of the HEXIM1 is found in the 7SK RNP complex and 13.5% is in the low molecular weight fraction. After induction through the TCR or by PMA treatment, the amount of HEXIM1 in the low molecular weight fraction more than doubles (29.8% TCR; 27.0% PMA).

Subthreshold levels of Tat contribute to HIV reactivation in latently infected cells

To evaluate whether P-TEFb released by TCR signaling in 2B2D cells required Tat in order to activate HIV transcription elongation we used ChIP assays to compare the HIV transcription elongation efficiencies in 2D10 and 2B2D cells at 0.5 hr, 2.5 hr and 5 hr after activation (Figure 10B). Consistent with the results described above, stimulation of both cells for 0.5 hr with TNF- α did not increase elongation efficiency above the basal level of $4.2 \pm 1.5\%$ in either cell line. However, activation of the TCR for 0.5 hr increased HIV elongation efficiency to 22.3% in the TCR stimulated 2D10 cells, but only to 6.5% in the 2B2D cells. Consistent with the results shown Figure 2C, at HIV elongation efficiency at 2.5 hr (15.0%) and 5.0 hr (48.4%) after activation by TNF- α there was a dramatic increase in due to the resumption of Tat synthesis from the reactivated provirus. There was only a modest further increase in elongation efficiency at 2.5 hr (25.0%) and 5.0 hr (27.7%) after TCR stimulation compared to the already high levels of elongation observed at 0.5 hr (22.3%). By contrast, in the 2B2D cells the elongation efficiency remains only slightly elevated with an average value of $6.2 \pm 0.6\%$ for the 0.5 hr, 2.5 hr and 5.0 hr time points.

Thus, the increase in HIV elongation efficiency that we have observed following TCR stimulation appears to require a combination of P-TEFb activation and the availability of Tat. Since as described above, there is no new synthesis of P-TEFb subunits following TCR activation, these data suggest that there is either activation of residual Tat activity in the 2D10 cells or there is new synthesis of Tat which does not occur after TNF- α stimulation (see Discussion).

In conclusion, our data demonstrates that functional P-TEFb can be released through an ERK-dependent cell signaling pathway. This previously uncharacterized signaling pathway represents one of the first examples of the physiological enhancement of P-TEFb activity and transcriptional elongation.

Discussion

Kinetics of HIV proviral reactivation

The strong conservation of a large number of *cis*-acting DNA elements in the HIV-1 LTR implies that the concerted action of numerous regulatory elements are needed to ensure robust production of viral mRNA in activated cells⁵⁵. In this paper we have begun to dissect how TCR-mediated signaling pathways contribute to the reactivation of latent HIV proviruses using the extensively characterized 2D10 cells as a model system^{25; 51}. A significant advantage of the 2D10 clone is that although it is highly restricted in unstimulated cells it is also readily inducible by a wide range of activators (Figure 1). Importantly, 2D10 cells, which are derived from the Jurkat T-cell clone E6, which was selected to carry a functional TCR signaling apparatus, are also highly responsive to activation by treatment with monoclonal antibodies directed against the CD3 receptor and CD28 co-activator. As shown here, stimulation of the TCR receptor activates an ordered program of transcription factor entry into the nucleus with early mobilization of NF- κ B and NFATc1 succeeded by mobilization of NFATc2.

Our experiments take advantage of the high degree of synchrony observed in T-cells following TCR activation which permits us to measure the oscillations in transcription activity that are associated with the rapid exchange of transcription factors between the nucleus and the cytoplasm. A second critical element in our studies is the use of quantitative ChIP assays to detect the distribution of RNAP II at various sites along the integrated HIV proviral genome. By comparing the concentration of RNAP II near the transcription start site to sites downstream we have been able to obtain accurate measurements of transcription initiation rates and elongation efficiencies. Because the ChIP assay directly measures RNAP II densities during transcription, it provides a much more accurate measurement of rapid changes in transcription than more traditional measurements based on the accumulation of a transcribed or translated product. Using these techniques we have been able to observe how periodic fluctuations in HIV transcription initiation on the order of 60 to 90 min (Figure 3) overlap with the progressive long-term changes in HIV transcription elongation efficiencies that occur due to the accumulation of Tat over an 8 hr time course (Figure 6).

A key conclusion arising from these analyses is that the first wave of HIV transcription initiation following TCR activation is driven primarily, if not exclusively, by NF- κ B. Although TCR activation also induces NFATc1 and NFATc2 which is fully functional and can support transcription of the NFAT-responsive genes such as EGR2 and TNF- α (Supplementary data), it is dispensable for HIV transcription since there is no significant change in RNAP II recruitment to the provirus when NFAT activation is blocked by treatment with cyclosporin A (Figure 1). Furthermore there were no measureable changes in HIV transcription initiation and elongation during the first 4 hr after TCR induction when cells are treated with cyclosporine (data not shown). This suggests that NF- κ B induced by TCR signaling effectively competes with NFAT for HIV LTR binding^{56; 57}.

Regulation of P-TEFb by ERK kinases enhances HIV transcriptional elongation

Although the first round of HIV transcription initiation induced by both TNF- α and TCR stimulation is mediated by NF- κ B, and RNAP II recruitment to the provirus shows nearly identical kinetics and levels under both conditions, dramatic differences in transcription elongation are evident even at the earliest times. As shown by multiple experiments, there is always an enhanced level of transcription elongation when cells are stimulated through the TCR compared to transcription elongation observed when cells are stimulated by TNF- α . This correlates with a dramatic increase in P-TEFb (CDK9) recruitment to the provirus, especially in downstream regions where elongation complexes accumulate (Figure 3). It is

important to note that the enhanced P-TEFb recruitment can be easily observed at 30 min, which is a time during the first phase of transcription prior to the accumulation of newly-synthesized Tat (see below).

Experiments using a variety of transcriptional activators and small molecule inhibitors of cell signaling pathways demonstrate that P-TEFb activation is mediated by ERK kinases (Figures 4 to 6). First, U0126, which is a potent inhibitor of ERK kinase activation, inhibits transcriptional elongation and P-TEFb recruitment to the HIV provirus in cells that have been activated either through the TCR or by PMA. Second, enhanced transcriptional elongation and P-TEFb recruitment can also be observed following activation of cells by PMA, a phorbol ester which activates protein kinase C and results in NF- κ B mobilization as well as activation of the ERK kinase pathway. Finally, treatment of cells with LY294002, which inhibits the AKT pathway, results in enhanced ERK phosphorylation following cell stimulation^{52; 53; 58} and a corresponding increase in transcription elongation and P-TEFb recruitment to HIV proviruses (Figures 4 & 6). Thus, we can correlate the extent of transcriptional elongation with the extent of ERK phosphorylation. Inhibitors such as U0126 that block ERK phosphorylation also block HIV transcription, whereas compounds that enhance ERK phosphorylation, such as LY294002, stimulate HIV transcription elongation and P-TEFb recruitment.

Direct evidence that P-TEFb is activated following TCR activation comes from experiments measuring the release of P-TEFb from the large 7SK RNP complex (Figures 7 to 9). Extensive disruption of the large complex is observed within 30 min of TCR stimulation. As expected, TCR-mediated 7SK RNP complex disruption can be blocked by treatment of cells with U0126 (Figure 9).

Our results are consistent with two earlier reports suggesting that either the ERK pathway or TCR signaling could activate P-TEFb. Fujita et al.⁵⁹ observed that thyrotropin-releasing hormone (TRH) can induce the recruitment of P-TEFb and transcription elongation to immediate early genes (c-fos, JunB, and MKP-1) in neuroendocrine GH4C1 cells. Importantly, as in our study, the ERK signaling pathway is activated by TRH in these cells and transcription elongation could be blocked by U0126. Second, while this work was in progress, Natarajan et al.⁵⁸ reported that TCR signaling can enhance HIV transcription by stimulating both NF- κ B and P-TEFb dissociation. While their conclusions are similar to ours, the two studies differ because Natarajan et al.⁵⁸ did not demonstrate directly that there was dissociation of the large 7SK RNP complex, nor did they show a direct effect of TCR signaling on HIV transcription elongation.

Regulation of P-TEFb by HMBA is distinct from activation of P-TEFb via the TCR

Previous studies have shown that disruption of P-TEFb complexes by HMBA can result in enhanced HIV transcriptional activity, however there have been conflicting claims about the mechanism of action of HMBA. Contreras et al.⁶⁰ have reported that HMBA can activate the PI3K/AKT pathway, which leads to the phosphorylation of HEXIM1 and the subsequent release of active pools of P-TEFb. However this mechanism does not appear to be responsible for the enhanced HIV transcription elongation that we have observed since inhibitors of the PI3K/AKT pathway such as AKT8 and LY294002 not only do not block HIV transcription but actually enhance it. An alternative mechanism for HMBA activation of HIV transcription was suggested by Choudhary et al.⁶¹, who reported that HMBA signaling is mediated via both protein kinase C α and PI3-kinase. Our observation that LY294002 stimulates HIV elongation also rules out this mechanism as responsible for the TCR-mediated activation of P-TEFb.

A third activation mechanism for activation of HIV transcription by HMBA has been reported by Chen et al.⁵⁴ who have reported that HMBA-induced release of P-TEFb from 7SK snRNP, is mediated by the calcium ion (Ca^{2+})-calmodulin-protein phosphatase 2B (PP2B) signaling pathway⁵⁴. In the mechanism that they described, Ca^{2+} signaling alone is insufficient to activate P-TEFb, and PP2B acts sequentially and cooperatively with protein phosphatase-1 α (PP1 α) which releases P-TEFb through dephosphorylating phospho-Thr186 in the CDK9 T-loop. Our data is most consistent with these findings. We have found that treatment of cells with HMBA for 30 min effectively disrupts the large 7SK RNP complex (Figure 8) but does not stimulate transcriptional elongation under these conditions (Figure 4), presumably because dephosphorylation of the T-loop of CDK9 inactivates the enzyme (Figure 9). Since HMBA can reactivate HIV proviruses over longer periods of time it is reasonable to assume that at some later stage the dephosphorylated enzyme released from the 7SK RNP complex can become rephosphorylated.

Tat-dependent and Tat-independent transcriptional elongation during HIV proviral reactivation

As shown in Figure 1, the HIV promoter is initially more restricted for transcriptional elongation than the promoter of the NF- κ B responsive gene *I κ B α* . As we will describe elsewhere, this additional restriction on elongation is due primarily to the enhanced recruitment of NELF to RNAP II initiating on latent proviruses due to the affinity of the NELF-E subunit for TAR RNA. As transcription proceeds, new Tat protein begins to accumulate after a lag of approximately 2 hr, and HIV transcriptional elongation efficiency becomes boosted to levels that are eventually over 3-fold higher than the levels observed on the NF- κ B-dependent cellular genes *I κ B α* and *JunB*.

During TCR signaling, dissociation of the large 7SK RNP complex increases the pool of active P-TEFb in the cell and enhances HIV transcriptional elongation. This results both in a global increase in the association of P-TEFb with chromatin (Figure 5) and increased HIV transcription. Activation of P-TEFb in response to TCR signaling does not require Tat since dissociation of the 7SK RNP complex can be observed in 2B2D cells²⁵ which harbor a latent provirus carrying the C22G mutation in Tat, which prevents its interactions with P-TEFb (Figure 10).

Tat levels in the latently infected 2D10 cells are extremely low and are below the level of detection by Western blotting (data not shown). Further evidence that there are only minimal Tat levels in latently infected cells comes from the observation that there is no HIV transcription in these cells above the basal activity seen in Tat-minus cells (Figure 10). Nonetheless, activation of P-TEFb via the TCR results in a disproportionate increase in HIV elongation at early time points in cells latently infected with proviruses carry that a functional Tat gene.

Where does this “new” P-TEFb:Tat activity come from? Our preferred hypothesis is that the latently infected 2D10 cells carry subthreshold levels of presynthesized Tat. We suggest that post-translational modification of components of the 7SK RNP complex through the TCR signaling pathway leads to the rapid disruption of the complex and increases the pool of active P-TEFb in the cell. The newly released P-TEFb is then able to associate with the residual Tat and enhance HIV transcriptional elongation. This hypothesis is based on the assumption that 15 to 30 min is an insufficient period of time to induce new HIV transcription and synthesize new Tat. For example, we have observed that following TNF- α activation of HIV transcription there is a lag period of approximately 2 hr prior to the enhancement of HIV transcription elongation which correlates closely with the kinetics of new Tat synthesis from latent proviruses reported by Williams et al.³³. An alternative hypothesis is that TCR signaling is able to induce new Tat synthesis in absence of new

mRNA synthesis. For example, it is possible that there is a residual pool of Tat mRNA that is restricted by miRNA or some other translational control mechanism that is regulated through the TCR. Although we are unable to formally rule out this hypothesis at this stage, we note that both Tat protein and Tat mRNA levels remain at extremely low levels during the first 2 hrs following TCR activation of the 2D10 cells and do not increase measurably (data not shown).

It remains an open question whether P-TEFb can ever be recruited to the HIV LTR in the absence of Tat. Recent studies of LPS-inducible primary response genes in macrophages have suggested that P-TEFb can be recruited to cellular promoters via Brd4 which both binds P-TEFb and can recognize chromatin carrying H4K5/8/12Ac markers⁶². However, this mechanism does not apply to latent HIV proviruses which characteristically show dramatically reduced acetylated histone levels compared to cellular primary response genes such as *IκBα*²⁵. The inability to efficiently recruit P-TEFb to HIV proviruses in the absence of Tat may be an important mechanism contributing to the maintenance of proviral latency.

Mobilization of P-TEFb in primary resting central memory T-cells

Stimulation of the T-cell receptor (TCR) by foreign antigens is believed to be the major physiological mechanism used to reactivate latent HIV proviruses. In addition to engagement of the TCR itself, several costimulatory molecules, including CD28, stimulate series of signaling cascades leading to T-cell proliferation, cytokine production and differentiation into effector cells. We have recently shown using a model for HIV latency in primary resting memory T-cells that TNF- α treatment efficiently activates NF- κ B but fails to activate P-TEFb. By contrast, TCR signaling induces both NF- κ B and P-TEFb²⁴ and is therefore able to activate latent HIV proviruses.

In contrast to Jurkat T-cells primary resting central memory T-cells show highly restricted levels of CycT1 and the presence of CDK9 that is inactive because of dephosphorylation of the critical Thr-186 in the T-loop⁴⁷. Activation of P-TEFb in these cells therefore requires multiple steps involving both the initial assembly of the 7SK RNP complex and its relocalization to nuclear speckles where it becomes accessible to the transcription machinery. It remains to be determined how the ERK-dependent signaling pathway we have identified in Jurkat T-cells contributes to the regulation of P-TEFb in the primary T-cells, however it is of interest to note that PMA can enhance CDK9 T-loop phosphorylation more than 10-fold in primary resting T-cells⁴⁷.

Therapeutic implications

There are currently extensive efforts being devoted devising “shock and kill” strategies for HIV eradication^{8; 63; 64}. In these strategies, a “shock” phase is used to reactivate latent proviruses, while a “kill” phase is used to eliminate the induced cells through immune responses, viral cytopathogenicity or cytotoxic drugs. In devising these strategies emphasis has been placed on using either histone deacetylase inhibitors, such as SAHA^{65; 66}, or activators of NF- κ B,^{67; 68} to reactivate the latent proviruses. Our observations that P-TEFb activation enhances the reactivation of latent proviruses suggests that effective activation of the entire latent viral pool may ultimately require cocktails of drugs that stimulate both transcription initiation and P-TEFb mobilization.

Materials and Methods

Materials

RPMI 1640 medium and fetal bovine serum (FBS) were purchased from Hyclone. Hexamethylene bisacetamide (HMBA), 5,6-dichlorobenzimidazole 1- β -ribofuranoside

(DRB), phytohemagglutinin (PHA), and LY294002 were from Sigma. U0126 was purchased from Calbiochem. α -CD3 and α -CD28 antibodies were from BD Biosciences. HEXIM-1 antibody was custom synthesized by Covance Research Products. Phospho-ERK 1/2 (Thr202/Tyr204), phospho-AKT (Thr308), and phospho-Cdk9 (Thr186) antibodies were from Cell Signaling. All other antibodies used in this study were purchased from Santa Cruz Biotechnology.

Cell culture and reactivation of clones

Isolation and reactivation of the 2D10 and 2B2D clones were described previously by Pearson et al.²⁵. 2D10 clone contains attenuated H13L Tat, and 2B2D has C22G mutation in Tat which is devoid of transactivation activity. Cells were maintained with RPMI 1640 supplemented with 10% FBS, penicillin (100 IU/ml), streptomycin (100 μ g/ml) and 25 mM HEPES at 37°C in 5% CO₂. The cells were reactivated with 10 ng/ml TNF- α ; 0.125 μ g/ml α -CD3 mAb plus 1 μ g/ml α -CD28 mAb; 0.125 μ g/ml α -CD3 antibody; 10 μ g/ml PHA; 50 ng/ml PMA; 50 ng/ml PMA plus 10 μ g/ml PHA; 500 nM TSA or 5 mM HMBA as indicated for 18 h and analyzed for d2EGFP expression by FACS.

Nuclear protein analysis

Nuclear protein was obtained as described by Hoffmann et al.⁶⁹. Briefly, 5×10^6 cells were collected and washed with ice-cold PBS. The cells were resuspended in CE lysis buffer (10 mM HEPES-KOH, 60 mM KCl, 1 mM EDTA, 0.5 % NP-40, 1mM DTT, 1mM PMSF) containing a cocktail of protease inhibitors (Roche) and incubated for 10 min on ice. Cells were vortexed and nuclei were pelleted by centrifugation for 10 min at 4000 rpm. Nuclei were resuspended in 60 μ l of NE buffer (250 mM Tris, pH 7.8, 60 mM KCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF) containing protease inhibitors and the nuclear proteins were extracted by several freeze and thaw cycles. Protein concentrations were determined using the BCA protein assay kit (Pierce) or Bradford's Reagent (Bio-Rad). The NuPAGE system (Invitrogen) was used to resolve proteins on a 10% Bis-Tris gel. Western blotting analysis was performed with antibodies against NF- κ B p65, NF- κ B p50, NFATc1, NFATc2, Spt5, phospho-ERK 1/2 (Thr202/Tyr 204), phospho-AKT (Thr308), and CDK9.

ChIP assay

2D10 cells were incubated with TNF- α (10 μ g/ml) or 0.125 μ g/ml α -CD3 mAb plus 1 μ g/ml α -CD28 mAb (TCR) for 0 h to 8 h. When indicated, 2D10 cells were preincubated for 1 h in the presence of inhibitors. 50 million cells were isolated from the population for each time point in the reactivation time course. Each sample was immediately fixed with 0.5 % of formaldehyde and prepared for ChIP as previously described (Kim et al). The antibodies used for immunoprecipitation were Total RNAP II (N-20, Santa Cruz Biotechnology) and CDK9 (H-169, Santa Cruz Biotechnology). Real-time PCR was performed by using 2% of each precipitated DNA. Percentage input for each data was determined by comparing the Ct value of each sample to a Ct standard curve generated from a serial dilution of genomic DNA. A no antibody control value was subtracted from each sample value to remove the nonspecific background signal. Primers used are as follows: HIV (promoter) -116 F- AGC TTG CTA CAA GGG ACT TTC C, HIV +4 R- ACC CAG TAC AGG CAA AAA GCA G. HIV (Nuc-1 position) +30 F- CTG GGA GCT CTC TGG CTA ACT A, HIV +134 R- TTA CCA GAG TCA CAC AAC AGA CG. HIV +283 F- GAC TGG TGA GTA CGC CAA AAA T, HIV +390 R- TTT CCC ATC GCG ATC TAA TTC. HIV +584 F- AGC AAC CCT CTA TTG T GT GCA T, +683 R- TGC GGT GGT CTT ACT TTT GTT T. HIV (env position) + 2593 F- TGA GGG ACA ATT GGA GAA GTG A, HIV +2691 R- TCT GCA CCA CTC TTC TCT TTG C. HIV (d2EGFP position) +4076 F- GAC AAG CAG AAG AAC GGC ATC, HIV +4172 R- GGG TGT TCT GCT GGT AGT GGT. IkB α +155 F-AAG AAG GAG CGG CTA CTG GAC and IkB α +237 R-TCC TTG ACC ATC TGC TCG TAC

T. I κ B α +2038 F- ATG CAG CCA TAA GCA TCT CAA A and I κ B α +2159 R- CCC ACA CTT CAA CAG GAG TGA C. I κ B α 2871 F- TGG TAG GAT CAG CCC TCA TTT T AND I κ B α 2978 R- AAC CCC ACA AAG GTG AGG TTT A.

Analysis of P-TEFb complexes in Jurkat cell lysates

Jurkat 2D10 or 2B2D cells were either untreated or treated for 30 min with either 0.125 μ g/ml α -CD3 mAb plus 1 μ g/ml α -CD28 mAb, 50 ng/ml PMA, 10 mM HMBA, or for 1 h with 100 μ M DRB. 5×10^7 cells were lysed and nuclear proteins extracted in cell lysis buffer A (10 mM HEPES, pH 8.0, 150 mM NaCl, 10 mM KCl, 1.5 mM MgCl₂, 0.5% NP-40, 1 mM DTT, 1 mM PMSF) containing a protease inhibitor cocktail, and where indicated, phosphatase inhibitors. Whole cell lysates were centrifuged at 5,000 rpm for 10 min and the supernatant was loaded onto a Superdex 200 resin in a Tricorn 10/300 sizing column. One bed volume of cell lysis buffer A was used for elution and 500 μ l fractions were collected. Methanol-chloroform precipitation was used to concentrate proteins in the collected fractions. The protein pellets corresponding to fractions 15 to 29 were resuspended under reducing conditions in 1X LDS sample buffer, heated at 95 °C for 10 min. then loaded onto a 10% Bis-Tris denaturing gel. Quantitative Western blotting analyses of the eluates were performed to examine the chromatography profiles of CDK9, phospho-Thr186 CDK9, and HEXIM-1.

P-TEFb association with chromatin

1×10^7 2D10 cells were stimulated by 10 ng/ml TNF- α (TNF) or 0.125 μ g/ml α -CD3 plus 1 μ g/ml α -CD28 mAb, collected and washed with ice-cold PBS. The cells were resuspended in hypotonic buffer (10mM KCl, 4mM MgCl₂, 10mM Hepes, pH 7.9, 2mM DTT, 750 mM spermidine, 150 mM spermine, and protease inhibitor cocktail) and then homogenized. Nuclei were pelleted and the supernatant collected as cytosolic extract (CE). The nuclei were incubated in a 1:1 solution of high salt buffer (0.72M KCl) and hypotonic buffer with periodic vortexing for 30 min at 4 °C and then pelleted by centrifugation to obtain the first extract (NE-1). The nuclear pellet was incubated as described above with NE buffer containing 360mM KCl to obtain the second extract (NE-2). The residual pellet was placed in 0.1% SDS buffer and sonicated. The solubilized fraction from the residual pellet was designated as NE-3. Protein concentrations were determined using the BCA protein assay kit (Pierce). The NuPAGE system (Invitrogen) was used to resolve 15 mg of total nuclear proteins on a 4–12% Bis-Tris gel. Western blotting analysis was performed with antibodies against CycT1, HEXIM1 and CDK9.

Data analysis

Flow cytometry data was analyzed using FloJo. Gates for the viable cells in the population (typically over 90% for unstimulated cells and over 80% for stimulated cells) were set based on the forward and side light scatter. Gates for d2EGFP were determined using uninfected Jurkat cells as a negative control and stimulated 2D10 cells as a positive control.

The Origin Pro (ver. 7.5) was used to generate curve fits for each of the data sets shown Figures 2 to 6 and Figures 8 and 9. The ChIP time course data sets shown in Figures 3 & 4 were fitted to a series of overlapping Gaussian distributions. To perform the fits, the baseline, y_0 , was fixed and 100 sequential iterations were used to find optimal parameters for the peak center, x_c , amplitude, A , and peak width, w , for each peak. The derivative of the original data set was used to objectively identify the number and approximate position of the peaks. To further refine the fits, x_c values for individual peaks were fixed in succession and values for the amplitude and peak width iteratively calculated until minimal χ^2 values were obtained.

Both the nuclear protein profiles (Figure 2) and the column chromatography data (Figure 10) were fitted using an analogous procedure to a series of overlapping skewed Gaussian functions of the form:

$$y=y_0+ Ae^{(-e^{-z}-z+1)}; z=(x-xc)/w$$

The data in Figure 6 was fitted either to the Boltzman distribution (Figure 6A & B) or to straight lines (Figure 6C & D). The data in Figure 8, showing the distribution of RNAP II and P-TEFb along the proviral genome was fitted to a single skewed Gaussian.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AFF4	AF4/FMR2 family member 4
AF9	protein AF9
AKT	Serine/threonine-protein kinase akt
AP-1	activator protein 1
BCDIN3	probable methyltransferase BCDIN3D
CDK9	cyclin dependent kinase 9
ChIP	chromatin immunoprecipitation
c-fos	proto-oncogene c-Fos
CsA	cyclosporine A
CTD	carboxyl terminal domain
CycT1	cyclin T1
d2EGFP	destabilized enhanced green fluorescent protein
DRB	5,6-dichlorobenzimidazole 1-β-ribofuranoside
DSIF	DRB sensitivity inducing factor
ELL2	RNA polymerase II elongation factor ELL2
ENL	protein ENL
ERK	extracellular-signal-regulated kinases
HAART	highly active antiretroviral treatment
HDAC	histone deacetylase

HEXIM	hexamethylene bis-acetamide inducible gene
HIV	human immunodeficiency virus
HMBA	hexamethylene bisacetamide
IκBα	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
JunB	Jun B proto-oncogene
LARP-7	La ribonucleoprotein domain family, member 7
mAb	monoclonal antibody
MAPK	mitogen-activated protein kinase
MAPKK	mitogen-activated protein kinase kinase
MKP-1	mitogen-activated protein kinase 1
NELF	negative elongation factor
NF-κB	nuclear factor kappa b
NFAT	nuclear factor of activated T-cells
PI3K	phosphatidylinositol 3-kinases
PHA	phytohemagglutinin
PMA	phorbol 12-myristate 13-acetate
PP2B	(Ca ²⁺)-calmodulin-protein phosphatase 2B
P-TEFb	positive transcription elongation factor b
RNAP II	RNA polymerase II
SAHA	suberoylanilide hydroxamic acid
SEPX1	selenoprotein X, 1
Spt5	Suppressor of Ty 5 homolog
Tat	trans-activator protein
Tatcom2	Tat complex 2
TAR	transactivation response region
TCR	T-cell receptor
TNF-α	Tumor necrosis factor alpha
TRH	thyrotropin-releasing hormone
TSA	trichostatin A
7SK RNP	small nuclear RNA 7SK ribonucleoprotein complex

References

1. Pierson T, McArthur J, Siliciano RF. Reservoirs for HIV-1: mechanisms for viral persistence in the presence of antiviral immune responses and antiretroviral therapy. *Annu Rev Immunol.* 2000; 18:665–708. [PubMed: 10837072]
2. Coiras M, Lopez-Huertas MR, Perez-Olmeda M, Alcamí J. Understanding HIV-1 latency provides clues for the eradication of long-term reservoirs. *Nat Rev Microbiol.* 2009; 7:798–812. [PubMed: 19834480]

3. Alexaki A, Liu Y, Wigdahl B. Cellular reservoirs of HIV-1 and their role in viral persistence. *Curr HIV Res.* 2008; 6:388–400. [PubMed: 18855649]
4. Chun TW, Davey RT Jr, Engel D, Lane HC, Fauci AS. Re-emergence of HIV after stopping therapy. *Nature.* 1999; 401:874–5. [PubMed: 10553903]
5. Davey RT Jr, Bhat N, Yoder C, Chun TW, Metcalf JA, Dewar R, Natarajan V, Lempicki RA, Adelsberger JW, Miller KD, Kovacs JA, Polis MA, Walker RE, Falloon J, Masur H, Gee D, Baseler M, Dimitrov DS, Fauci AS, Lane HC. HIV-1 and T cell dynamics after interruption of highly active antiretroviral therapy (HAART) in patients with a history of sustained viral suppression. *Proc Natl Acad Sci U S A.* 1999; 96:15109–14. [PubMed: 10611346]
6. Fischer M, Hafner R, Schneider C, Trkola A, Joos B, Joller H, Hirschel B, Weber R, Gunthard HF. HIV RNA in plasma rebounds within days during structured treatment interruptions. *AIDS.* 2003; 17:195–9. [PubMed: 12545079]
7. Dinoso JB, Kim SY, Wiegand AM, Palmer SE, Gange SJ, Cranmer L, O'Shea A, Callender M, Spivak A, Brennan T, Kearney MF, Proschan MA, Mican JM, Rehm CA, Coffin JM, Mellors JW, Siliciano RF, Maldarelli F. Treatment intensification does not reduce residual HIV-1 viremia in patients on highly active antiretroviral therapy. *Proc Natl Acad Sci U S A.* 2009; 106:9403–8. [PubMed: 19470482]
8. Richman DD, Margolis DM, Delaney M, Greene WC, Hazuda D, Pomerantz RJ. The challenge of finding a cure for HIV infection. *Science.* 2009; 323:1304–7. [PubMed: 19265012]
9. Shen L, Siliciano RF. Viral reservoirs, residual viremia, and the potential of highly active antiretroviral therapy to eradicate HIV infection. *J Allergy Clin Immunol.* 2008; 122:22–8. [PubMed: 18602567]
10. Trono D, Van Lint C, Rouzioux C, Verdin E, Barre-Sinoussi F, Chun TW, Chomont N. HIV persistence and the prospect of long-term drug-free remissions for HIV-infected individuals. *Science.* 2010; 329:174–80. [PubMed: 20616270]
11. Peterlin BM, Price DH. Controlling the elongation phase of transcription with P-TEFb. *Mol Cell.* 2006; 23:297–305. [PubMed: 16885020]
12. Karn J. The molecular biology of HIV latency: breaking and restoring the Tat-dependent transcriptional circuit. *Curr Opin HIV and AIDS.* 2011; 6:4–11.
13. Singh A, Weinberger LS. Stochastic gene expression as a molecular switch for viral latency. *Curr Opin Microbiol.* 2009; 12:460–6. [PubMed: 19595626]
14. Wei P, Garber ME, Fang SM, Fischer WH, Jones KA. A novel cdk9-associated c-type cyclin interacts directly with HIV-1 Tat and mediates its high-affinity, loop specific binding to TAR RNA. *Cell.* 1998; 92:451–462. [PubMed: 9491887]
15. Fujinaga K, Irwin D, Huang Y, Taube R, Kurosu T, Peterlin BM. Dynamics of human immunodeficiency virus transcription: P-TEFb phosphorylates RD and dissociates negative effectors from the transactivation response element. *Mol Cell Biol.* 2004; 24:787–795. [PubMed: 14701750]
16. Kim YK, Bourgeois CF, Isel C, Churcher MJ, Karn J. Phosphorylation of the RNA polymerase II carboxyl-terminal domain by CDK9 is directly responsible for human immunodeficiency virus type 1 Tat-activated transcriptional elongation. *Mol Cell Biol.* 2002; 22:4622–4637. [PubMed: 12052871]
17. Bourgeois CF, Kim YK, Churcher MJ, West MJ, Karn J. Spt5 cooperates with Tat by preventing premature RNA release at terminator sequences. *Mol Cell Biol.* 2002; 22:1079–1093. [PubMed: 11809800]
18. Ivanov D, Kwak YT, Guo J, Gaynor RB. Domains in the SPT5 protein that modulate its transcriptional regulatory properties. *Mol Cell Biol.* 2000; 20:2970–2983. [PubMed: 10757782]
19. Weinberger LS, Burnett JC, Toettcher JE, Arkin AP, Schaffer DV. Stochastic gene expression in a lentiviral positive-feedback loop: HIV-1 Tat fluctuations drive phenotypic diversity. *Cell.* 2005; 122:169–82. [PubMed: 16051143]
20. Weinberger LS, Dar RD, Simpson ML. Transient-mediated fate determination in a transcriptional circuit of HIV. *Nat Genet.* 2008; 40:466–70. [PubMed: 18344999]

21. Keedy KS, Archin NM, Gates AT, Espeseth A, Hazuda DJ, Margolis DM. A limited group of class I histone deacetylases act to repress human immunodeficiency virus type-1 expression. *J Virol.* 2009; 83:4749–4756. [PubMed: 19279091]
22. Williams SA, Chen LF, Kwon H, Ruiz-Jarabo CM, Verdin E, Greene WC. NF-kappaB p50 promotes HIV latency through HDAC recruitment and repression of transcriptional initiation. *EMBO J.* 2006; 25:139–149. [PubMed: 16319923]
23. Tyagi M, Karn J. CBF-1 promotes transcriptional silencing during the establishment of HIV-1 latency. *EMBO J.* 2007; 26:4985–95. [PubMed: 18007589]
24. Tyagi M, Pearson RJ, Karn J. Establishment of HIV latency in primary CD4+ cells is due to epigenetic transcriptional silencing and P-TEFb restriction. *J Virol.* 2010; 84:6425–37. [PubMed: 20410271]
25. Pearson R, Kim YK, Hokello J, Lassen K, Friedman J, Tyagi M, Karn J. Epigenetic silencing of human immunodeficiency virus (HIV) transcription by formation of restrictive chromatin structures at the viral long terminal repeat drives the progressive entry of HIV into latency. *J Virol.* 2008; 82:12291–303. [PubMed: 18829756]
26. du Chene I, Basyuk E, Lin YL, Triboulet R, Knezevich A, Chable-Bessia C, Mettling C, Baillat V, Reynes J, Corbeau P, Bertrand E, Marcello A, Emiliani S, Kiernan R, Benkirane M. Suv39H1 and HP1gamma are responsible for chromatin-mediated HIV-1 transcriptional silencing and post-integration latency. *EMBO J.* 2007; 26:424–35. [PubMed: 17245432]
27. Marban C, Suzanne S, Dequiedt F, de Walque S, Redel L, Van Lint C, Aunis D, Rohr O. Recruitment of chromatin-modifying enzymes by CTIP2 promotes HIV-1 transcriptional silencing. *EMBO J.* 2007; 26:412–23. [PubMed: 17245431]
28. Blazkova J, Trejbalova K, Gondoys-Rey F, Halfon P, Philibert P, Guiguen A, Verdin E, Olive D, Van Lint C, Hejnar J, Hirsch I. CpG methylation controls reactivation of HIV from latency. *PLoS Pathog.* 2009; 5:e1000554. [PubMed: 19696893]
29. Kauder SE, Bosque A, Lindqvist A, Planelles V, Verdin E. Epigenetic regulation of HIV-1 latency by cytosine methylation. *PLoS Pathog.* 2009; 5:e1000495. [PubMed: 19557157]
30. Han Y, Lin YB, An W, Xu J, Yang HC, O'Connell K, Dordai D, Boeke JD, Siliciano JD, Siliciano RF. Orientation-dependent regulation of integrated HIV-1 expression by host gene transcriptional readthrough. *Cell Host Microbe.* 2008; 4:134–46. [PubMed: 18692773]
31. Lenasi T, Contreras X, Peterlin BM. Transcriptional interference antagonizes proviral gene expression to promote HIV latency. *Cell Host Microbe.* 2008; 4:123–33. [PubMed: 18692772]
32. Duverger A, Jones J, May J, Bibollet-Ruche F, Wagner FA, Cron RQ, Kutsch O. Determinants of the establishment of human immunodeficiency virus type 1 latency. *J Virol.* 2009; 83:3078–93. [PubMed: 19144703]
33. Williams SA, Kwon H, Chen LF, Greene WC. Sustained induction of NF-kappaB is required for efficient expression of latent human immunodeficiency virus type 1. *J Virol.* 2007; 81:6043–56. [PubMed: 17376917]
34. Bosque A, Planelles V. Induction of HIV-1 latency and reactivation in primary memory CD4+ T cells. *Blood.* 2008; 113:58–65. [PubMed: 18849485]
35. Nabel G, Baltimore DA. An inducible transcription factor activates expression of human immunodeficiency virus in T cells. *Nature.* 1987; 326:711–713. [PubMed: 3031512]
36. Kinoshita S, Su L, Amano M, Timmerman LA, Kaneshima H, Nolan GP. The T cell activation factor NF-ATc positively regulates HIV-1 replication and gene expression in T cells. *Immunity.* 1997; 6:235–244. [PubMed: 9075924]
37. Nguyen VT, Kiss T, Michels AA, Bensaude O. 7SK small nuclear RNA binds to and inhibits the activity of CDK9/cyclin T complexes. *Nature.* 2001; 414:322–325. [PubMed: 11713533]
38. Yang Z, Zhu Q, Luo K, Zhou Q. The 7SK small nuclear RNA inhibits the CDK9/cyclin T1 kinase to control transcription. *Nature.* 2001; 414:317–322. [PubMed: 11713532]
39. Yik JH, Chen R, Nishimura R, Jennings JL, Link AJ, Zhou Q. Inhibition of P-TEFb (CDK9/Cyclin T) kinase and RNA polymerase II transcription by the coordinated actions of HEXIM1 and 7SK snRNA. *Mol Cell.* 2003; 12:971–82. [PubMed: 14580347]

40. Michels AA, Fraldi A, Li Q, Adamson TE, Bonnet F, Nguyen VT, Sedore SC, Price JP, Price DH, Lania L, Bensaude O. Binding of the 7SK snRNA turns the HEXIM1 protein into a P-TEFb (CDK9/cyclin T) inhibitor. *EMBO J.* 2004; 23:2608–2619. [PubMed: 15201869]
41. He N, Jahchan NS, Hong E, Li Q, Bayfield MA, Maraia RJ, Luo K, Zhou Q. A La-related protein modulates 7SK snRNP integrity to suppress P-TEFb-dependent transcriptional elongation and tumorigenesis. *Mol Cell.* 2008; 29:588–99. [PubMed: 18249148]
42. Krueger BJ, Jeronimo C, Roy BB, Bouchard A, Barrandon C, Byers SA, Searcey CE, Cooper JJ, Bensaude O, Cohen EA, Coulombe B, Price DH. LARP7 is a stable component of the 7SK snRNP while P-TEFb, HEXIM1 and hnRNP A1 are reversibly associated. *Nucleic Acids Res.* 2008; 36:2219–29. [PubMed: 18281698]
43. Jeronimo C, Forget D, Bouchard A, Li Q, Chua G, Poitras C, Therien C, Bergeron D, Bourassa S, Greenblatt J, Chabot B, Poirier GG, Hughes TR, Blanchette M, Price DH, Coulombe B. Systematic analysis of the protein interaction network for the human transcription machinery reveals the identity of the 7SK capping enzyme. *Mol Cell.* 2007; 27:262–74. [PubMed: 17643375]
44. Barboric M, Yik JH, Czudnochowski N, Yang Z, Chen R, Contreras X, Geyer M, Matija Peterlin B, Zhou Q. Tat competes with HEXIM1 to increase the active pool of P-TEFb for HIV-1 transcription. *Nucleic Acids Res.* 2007; 35:2003–12. [PubMed: 17341462]
45. Sedore SC, Byers SA, Biglione S, Price JP, Maury WJ, Price DH. Manipulation of P-TEFb control machinery by HIV: recruitment of P-TEFb from the large form by Tat and binding of HEXIM1 to TAR. *Nucleic Acids Res.* 2007; 35:4347–58. [PubMed: 17576689]
46. Krueger BJ, Varzavand K, Cooper JJ, Price DH. The mechanism of release of P-TEFb and HEXIM1 from the 7SK snRNP by viral and cellular activators includes a conformational change in 7SK. *PLoS One.* 2010; 5:e12335. [PubMed: 20808803]
47. Ramakrishnan R, Dow EC, Rice AP. Characterization of Cdk9 T-loop phosphorylation in resting and activated CD4(+) T lymphocytes. *J Leukoc Biol.* 2009; 86:1345–50. [PubMed: 19741158]
48. Dow EC, Liu H, Rice AP. T-loop phosphorylated Cdk9 localizes to nuclear speckle domains which may serve as sites of active P-TEFb function and exchange between the Brd4 and 7SK/HEXIM1 regulatory complexes. *J Cell Physiol.* 2010; 224:84–93. [PubMed: 20201073]
49. Sung TL, Rice AP. miR-198 inhibits HIV-1 gene expression and replication in monocytes and its mechanism of action appears to involve repression of cyclin T1. *PLoS Pathog.* 2009; 5:e1000263. [PubMed: 19148268]
50. Kim YK, Bourgeois CF, Pearson R, Tyagi M, West MJ, Wong J, Wu SY, Chiang CM, Karn J. Recruitment of TFIID to the HIV LTR is a rate-limiting step in the emergence of HIV from latency. *EMBO J.* 2006; 25:3596–604. [PubMed: 16874302]
51. Meredith LW, Sivakumaran H, Major L, Suhrbier A, Harrich D. Potent inhibition of HIV-1 replication by a Tat mutant. *PLoS One.* 2009; 4:e7769. [PubMed: 19901984]
52. Guan KL, Figueroa C, Brtva TR, Zhu T, Taylor J, Barber TD, Vojtek AB. Negative regulation of the serine/threonine kinase B-Raf by Akt. *J Biol Chem.* 2000; 275:27354–9. [PubMed: 10869359]
53. Zimmermann S, Moelling K. Phosphorylation and regulation of Raf by Akt (protein kinase B). *Science.* 1999; 286:1741–4. [PubMed: 10576742]
54. Chen R, Liu M, Li H, Xue Y, Ramey WN, He N, Ai N, Luo H, Zhu Y, Zhou N, Zhou Q. PP2B and PP1alpha cooperatively disrupt 7SK snRNP to release P-TEFb for transcription in response to Ca²⁺ signaling. *Genes Dev.* 2008; 22:1356–68. [PubMed: 18483222]
55. Pereira LA, Bentley K, Peeters A, Churchill MJ, Deacon NJ. A compilation of cellular transcription factor interactions with the HIV-1 LTR promoter. *Nucleic Acids Res.* 2000; 28:663–8. [PubMed: 10637316]
56. Chen-Park FE, Huang DB, Noro B, Thanos D, Ghosh G. The κB DNA sequence from the HIV long terminal repeat functions as an allosteric regulator of HIV transcription. *J Biol Chem.* 2002; 277:24701–8. [PubMed: 11970949]
57. Giffin MJ, Stroud JC, Bates DL, von Koenig KD, Hardin J, Chen L. Structure of NFAT1 bound as a dimer to the HIV-1 LTR κB element. *Nature Struct Biol.* 2003; 10:800–806. [PubMed: 12949493]

58. Natarajan M, August A, Henderson AJ. Combinatorial signals from CD28 differentially regulate human immunodeficiency virus transcription in T cells. *J Biol Chem*. 2010; 285:17338–47. [PubMed: 20368329]
59. Fujita T, Ryser S, Piuze I, Schlegel W. Up-regulation of P-TEFb by the MEK1-ERK signaling pathway contributes to stimulated transcription elongation of immediate early genes in neuroendocrine cells. *Mol Cell Biol*. 2008; 28:1630–43. [PubMed: 18086894]
60. Contreras X, Barboric M, Lenasi T, Peterlin BM. HMBA releases P-TEFb from HEXIM1 and 7SK snRNA via PI3K/Akt and activates HIV transcription. *PLoS Pathog*. 2007; 3:1459–69. [PubMed: 17937499]
61. Choudhary SK, Archin NM, Margolis DM. Hexamethylbisacetamide and disruption of human immunodeficiency virus type 1 latency in CD4(+) T cells. *J Infect Dis*. 2008; 197:1162–70. [PubMed: 18419522]
62. Hargreaves DC, Horng T, Medzhitov R. Control of inducible gene expression by signal-dependent transcriptional elongation. *Cell*. 2009; 138:129–145. [PubMed: 19596240]
63. Savarino A, Mai A, Norelli S, El Daker S, Valente S, Rotili D, Altucci L, Palamara AT, Garaci E. “Shock and kill” effects of class I-selective histone deacetylase inhibitors in combination with the glutathione synthesis inhibitor buthionine sulfoximine in cell line models for HIV-1 quiescence. *Retrovirology*. 2009; 6:52. [PubMed: 19486542]
64. Bowman MC, Archin NM, Margolis DM. Pharmaceutical approaches to eradication of persistent HIV infection. *Expert Rev Mol Med*. 2009; 11:e6. [PubMed: 19208267]
65. Archin NM, Espeseth A, Parker D, Cheema M, Hazuda D, Margolis DM. Expression of latent HIV induced by the potent HDAC inhibitor suberoylanilide hydroxamic acid. *AIDS Res Hum Retroviruses*. 2009; 25:207–12. [PubMed: 19239360]
66. Contreras X, Schweneker M, Chen CS, McCune JM, Deeks SG, Martin J, Peterlin BM. Suberoylanilide hydroxamic acid reactivates HIV from latently infected cells. *J Biol Chem*. 2009; 284:6782–9. [PubMed: 19136668]
67. Williams SA, Chen LF, Kwon H, Fenard D, Bisgrove D, Verdin E, Greene WC. Prostratin antagonizes HIV latency by activating NF-kappaB. *J Biol Chem*. 2004; 279:42008–17. [PubMed: 15284245]
68. Yang HC, Xing S, Shan L, O’Connell K, Dinoso J, Shen A, Zhou Y, Shrum CK, Han Y, Liu JO, Zhang H, Margolick JB, Siliciano RF. Small-molecule screening using a human primary cell model of HIV latency identifies compounds that reverse latency without cellular activation. *J Clin Invest*. 2009; 119:3473–86. [PubMed: 19805909]
69. Hoffmann A, Levchenko A, Scott ML, Baltimore D. The I κ B-NF- κ B signaling module: temporal control and selective gene activation. *Science*. 2002; 298:1241–1245. [PubMed: 12424381]

Figure 1. Induction of latently infected 2D10 cells by stimulation of the TCR

(A) Analysis of HIV proviral activation by flow cytometry. Cells were activated with 10 ng/ml TNF- α (left panels) or α -CD3 mAb plus α -CD28 mAb (right panels) in the presence or absence of CsA. Note that CsA did not prevent proviral reactivation following TCR signaling. (B) Nuclear protein levels of the NF- κ B subunits p65 and p50 and NFAT family members NFATc1 and NFATc2 were monitored during a 6 hr time course. Western blot of nuclear proteins following stimulation of 2D10 cells with 10 ng/ml TNF- α (left panels) or 0.125 μ g/ml α -CD3 mAb plus 1 μ g/ml α -CD28 mAb (right panels).

Figure 2. Stimulation of HIV transcriptional elongation following TNF- α stimulation and T cell receptor-mediated activation of latent proviruses

Chromatin immunoprecipitation (ChIP) assays performed using RNAP II antibodies (N-20, Santa Cruz Biotechnology) were used to measure RNAP II distributions on the HIV provirus and the I κ B α gene in clone 2D10 cells. (A) HIV transcription. Left panel: RNAP II levels at the transcription start site (HIV +30 to +134 region). Throughout this figure ChIP data sets were fitted to series of overlapping Gaussian distributions as described in the Methods. Middle panel: RNAP II levels at a downstream region (HIV +2593 to +2691). Right Panel: HIV elongation efficiency as measured by the ratio of RNAP II at +2593 and +30. The data was fitted to a biphasic curve. (B) I κ B α gene elongation efficiency as measured by the ratio of RNAP II levels at +2038 to +155. Left panel: RNAP II levels near the transcription start site (I κ B α +155 to +237 region). Middle panel: RNAP II levels at a downstream region (I κ B α +2038 to +2159). Right Panel: I κ B α elongation efficiency as measured by the ratio of RNAP II at +2038 and +155. The data was fitted to a straight line. Blue lines and squares: activation of 2D10 cells with 10 ng/ml TNF- α . Red lines and circles: Activation of 2D10 cells with 0.125 μ g/ml α -CD3 mAb plus 1 μ g/ml α -CD28 mAb. Note that activation through the TCR results in more than twice the amount of RNAP II reaching the downstream regions of the HIV provirus than is seen following activation by TNF- α .

Figure 3. Enhanced recruitment of P-TEFb to HIV proviruses following TCR activation
ChIP assays were used to measure the distribution of RNAP II and CDK9 at multiple locations along the HIV provirus. (A) RNAP II distributions. Left: 2D10 cells induced with 10 ng/ml TNF- α for 30 min (light green circle) and uninduced controls (dark green squares). Right: Cells induced for 30 min with 0.125 μ g/ml α -CD3 mAb plus 1 μ g/ml α -CD28 mAb (red circles) and uninduced controls (orange squares). (B) CDK9 distributions from the same experiments as shown in panel A. Note that activation of cells through the TCR simultaneously stimulates P-TEFb recruitment and RNAP II elongation, but TNF- α stimulation has only minimal effects on the P-TEFb recruitment.

Figure 4. ERK kinase pathway mediates TCR activation of HIV transcriptional elongation

(A) Western blots showing ERK and AKT phosphorylation following TCR activation. Left panels: Suppression of ERK phosphorylation by U0126. 2D10 cells were induced for 30 min with 0.125 μ g/ml α -CD3 mAb plus 1 μ g/ml α -CD28 mAb in presence or absence of 10 μ M U0126 and a combination of 10 μ M U0126 and 10 μ M LY294002. Nuclear and cytoplasmic extracts were prepared and blotted using antibodies against phosphorylated ERK-1/2 and phosphorylated AKT (Cell Signaling Technology). The nuclear protein Spt5 was included as a loading control. Right panels: Activation of ERK phosphorylation by LY294002. Cells were induced for 30 min with 0.125 μ g/ml α -CD3 mAb plus 1 μ g/ml α -CD28 mAb in presence or absence of 10 μ M LY294002 and a combination of 10 μ M U0126 and 10 μ M LY294002 and blotted as described above. (B) Chromatin immunoprecipitation assays to measure the elongation efficiency of RNAP II on induced HIV proviruses. Left panels: 2D10 cells were stimulated for 30 min with 5 ng/ml TNF- α (TNF); 0.125 μ g/ml α -CD3 mAb (CD3); 0.125 μ g/ml α -CD3 plus 1 μ g/ml α -CD28 mAb, CD3 antibody (TCR); 10 μ g/ml PHA (PHA); 50 ng/ml PMA (PMA); 50 ng/ml PMA plus 10 μ g/ml PHA; 500 nM TSA (TSA); 5 mM HMBA. Center panels: Control 2D10 cells or cells activated with 10 ng/ml TNF- α or 0.125 μ g/ml α -CD3 plus 1 μ g/ml α -CD28 mAb for 30 min. The cells were pretreated for 1 h as follows: 10 μ M LY294002 (LY); 1 μ M Akt inhibitor 8 (AI8); 10 μ M

U0126 (U0126), or 1 μ g/ml Cyclosporin A (CsA). Right panels: Cells activated with 10 ng/ml TNF- α (TNF- α) or 50 ng/ml PMA (PMA) for 30 min. The cells were pretreated for 1 h with either 20 μ M U0126 or 10 μ M LY294002. Red bars: RNAP II levels at the transcription start site (+30 to +134) and downstream region (+4076 to +4172). Blue bars: Elongation efficiency as measured by the ratio of the downstream to upstream RNAP II levels. Broken line indicates the average elongation efficiency of unstimulated or TNF- α stimulated cells. Note that U0126 strongly inhibited stimulation of HIV elongation induced by TCR and PMA stimulation whereas LY294002 enhanced HIV elongation.

Figure 5. P-TEFb association with chromatin is enhanced following TCR activation in the absence of new P-TEFb subunit synthesis

(A) Western blot showing nuclear levels of p65, ERK-1, phosphorylated ERK-1,2 (pERK-1/2) and Spt5 following TCR activation. Left panels: 2D10 cells were stimulated for up to 60 min with 0.125 μ g/ml α -CD3 plus 1 μ g/ml α -CD28 mAb, CD3 antibody (TCR). Right panels: Cells stimulated in the presence of 20 μ M U0126. Note that TCR stimulation strongly increases the levels of pERK-1/2 but does not alter the total levels of nuclear ERK-1. (B) Western blot showing nuclear levels of p65, CDK9, CycT1 and CDK7 following activation through the TCR (left panels) or by 10 ng/ml TNF- α (right panels). Note that the nuclear levels of CDK9 and CycT1 are unaltered compared to the CDK7 control. (C) TCR signaling enhances the association of P-TEFb with chromatin. 2D10 cells were treated for 60 min with 0.125 μ g/ml α -CD3 plus 1 μ g/ml α -CD28 mAb, CD3 antibody (TCR), or 100 mM DRB, or 1 μ g/ml actinomycin D (Act D). Nuclei were prepared and extracted as described in the Materials and methods. The cytoplasmic and nuclear extracts were examined by Western blotting using antibodies to CycT1, HEXIM1 and CDK9.

Figure 6. U0126 blocks TCR mediated HIV transcription elongation

(A) Western blot showing accumulation of the transcription factors NF- κ B and NFAT in the nucleus after activation of cells with either 0.125 μ g/ml α -CD3 mAb plus 1 μ g/ml α -CD28 mAb, either in the absence of U0126 or after pretreatment with 10 μ M U0126 for 1 h. Included in the blot are analyses of the nuclear levels of ERK 1/2 and phospho-ERK. (B) The distribution of RNAP II on the HIV provirus measured by ChIP assays following TCR activation. U0126 had little effects on RNAP levels upstream (+30 to +134), but it significantly reduced the amount of RNAP downstream (+2593 to 2691).

Figure 7. Recruitment of P-TEFb to HIV proviruses following PMA activation is blocked by U0126 and enhanced by LY294002

ChIP assays were used to measure the distribution of RNAP II and CDK9 at multiple locations along HIV provirus genome. (A) RNAP II distributions. Left: Cells induced for 30 min with 50 ng/ml PMA (red circles), cells induced with PMA in the presence of 20 μ M U0126 (yellow triangles) and uninduced controls (black squares). Right: Cells induced for 30 min with 50 ng/ml PMA and 10 μ M LY294002 (purple circles), cells induced with PMA and LY294002 in the presence of 20 μ M U0126 (light blue triangles) and uninduced controls (dark blue squares). (B) CDK9 distributions from the same experiments as shown in panel A.

Figure 8. TCR signaling induces the disassembly of the 7SK RNP complex

Whole cell lysates were fractionated on Superdex 200 gel filtration columns. Quantitative Western blotting analysis was performed to examine the chromatography profiles of CDK9 (left) and HEXIM-1 (right). Top panels: Western blots. Bottom panels: Elution profiles based on densitometry analysis of the western blots. (A) Disruption of 7SK complexes by TCR signaling. Jurkat 2D10 cells were treated for 30 min with 0.125 $\mu\text{g/ml}$ $\alpha\text{-CD3}$ mAb plus 1 $\mu\text{g/ml}$ $\alpha\text{-CD28}$ mAb. (B) Disruption by 10 mM HMBA, or for 1 h with 100 μM DRB.

Figure 9. The ERK pathway mediates the disassembly of the 7SK RNP complex in response to TCR activation

Whole cell lysates were fractionated on Superdex 200 gel filtration columns. Quantitative Western blotting analysis was performed to examine the chromatography profiles of CDK9 (left) and HEXIM-1 (right). Top panels: Western blots. Bottom panels: Elution profiles based on densitometry analysis of the western blots. (A) U0126 blocks disruption of the 7SK complex induced by TCR activation. Jurkat 2D10 cells were treated for 30 min with 0.125 $\mu\text{g/ml}$ $\alpha\text{-CD3}$ mAb plus 1 $\mu\text{g/ml}$ $\alpha\text{-CD28}$ mAb with or without 1 h pretreatment with 10 μM U0126. Panel shows Western blotting analysis and chromatography profiles of CDK9 (left) and HEXIM-1 (right). (B) CDK9 present in P-TEFb released by TCR activation remains phosphorylated on the T-loop. Jurkat 2D10 cells were treated for 30 min with either $\alpha\text{-CD3}$ / $\alpha\text{-CD28}$ antibodies, or 5 mM HMBA. Panel shows Western blotting analysis and chromatography profiles of CDK9 (left) and phospho-Thr 186 CDK9 (right).

Figure 10. Disruption of the 7SK RNP complex in response to TCR activation occurs in the absence of HIV Tat

(A) Disruption of 7SK RNP complex in Tat minus (2B2D) cells. Whole cell lysates were fractionated on Superdex 200 gel filtration columns and quantitative Western blotting analysis was performed to examine the chromatography profiles of CDK9 (left) and HEXIM-1 (right). Top panels: Western blots. Bottom panels: Elution profiles based on densitometry analysis of the western blots. (B) HIV transcription elongation efficiency. Chip assays were performed as described in legend to Figure 4B. Left panels: 2D10 cells were stimulated for 0.5 hr, 2.5 hr or 10 hr with either 5 ng/ml TNF- α (TNF) or 0.125 μ g/ml α -CD3 plus 1 μ g/ml α -CD28 mAb, CD3 antibody (TCR). Right panels: 2B2D cells were stimulated for 0.5 hr, 2.5 hr or 10 hr with either 5 ng/ml TNF- α (TNF) or 0.125 μ g/ml α -CD3 plus 1 μ g/ml α -CD28 mAb, CD3 antibody (TCR). Top panels, red bars: RNAP II levels at the transcription start site (+30 to +134). Top panels, black bars: downstream region (+4076 to +4172). Bottom panels, blue bars: Elongation efficiency as measured by the ratio of the downstream to upstream RNAP II levels. Broken line indicates the average elongation efficiency of unstimulated or TNF- α stimulated cells.