

Manipulation of P-TEFb control machinery by HIV: recruitment of P-TEFb from the large form by Tat and binding of HEXIM1 to TAR

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ABSTRACT

Basal transcription of the HIV LTR is highly repressed and requires Tat to recruit the positive transcription elongation factor, P-TEFb, which functions to promote the transition of RNA polymerase II from abortive to productive elongation. P-TEFb is found in two forms in cells, a free, active form and a large, inactive complex that also contains 7SK RNA and HEXIM1 or HEXIM2. Here we show that HIV infection of cells led to the release of P-TEFb from the large form. Consistent with Tat being the cause of this effect, transfection of a FLAG-tagged Tat in 293T cells caused a dramatic shift of P-TEFb out of the large form to a smaller form containing Tat. *In vitro*, Tat competed with HEXIM1 for binding to 7SK, blocked the formation of the P-TEFb–HEXIM1–7SK complex, and caused the release P-TEFb from a pre-formed P-TEFb–HEXIM1–7SK complex. These findings indicate that Tat can acquire P-TEFb from the large form. In addition, we found that HEXIM1 binds tightly to the HIV 5' UTR containing TAR and recruits and inhibits P-TEFb activity. This suggests that in the absence of Tat, HEXIM1 may bind to TAR and repress transcription elongation of the HIV LTR.

INTRODUCTION

The positive transcription elongation factor, P-TEFb, is an essential cellular transcription factor that controls the transition of RNA polymerase II from abortive to productive elongation (1–3). P-TEFb is composed of one of two isoforms of the cyclin-dependent kinase Cdk9 (4,5) and a partner cyclin, T1, T2 or K (6,7). P-TEFb phosphorylates serine 2 residues in the heptapeptide repeat present in the carboxyl terminal domain (CTD) of the largest subunit of RNA polymerase II (5–7) and

DRB Sensitivity Inducing Factor (DSIF) (8,9), alleviating the effect of the negative elongation factors DSIF and Negative ELongation Factor (NELF) (10–12). Knockdown of P-TEFb subunits by RNAi in *Caenorhabditis elegans* reduces serine 2 phosphorylation in the CTD and the expression of early embryonic genes, and results in an embryonic lethal phenotype (13). Inhibition of P-TEFb activity in cells through treatment with the P-TEFb inhibitor flavopiridol, blocks most RNA polymerase II transcription, and extended treatment leads to cell death (14).

P-TEFb was recently found to be regulated by reversible inhibition via association with the small nuclear RNA 7SK (15,16) and the RNA-binding proteins HEXIM1 (17–20) or HEXIM2 (21,22). Association of 7SK with a HEXIM1 dimer relieves an autoinhibitory interaction between two regions of HEXIM1, allowing recruitment and inhibition of two P-TEFb molecules (22–25). The large form of P-TEFb contains potentially active P-TEFb molecules, because the activating T-loop phosphorylation of Cdk9 is required for its incorporation into the large form (25,26). While the exact signals and mechanisms for mobilization of P-TEFb from the large form remain undefined, the association of P-TEFb with 7SK and the HEXIM proteins is highly responsive to intra- and extracellular signaling. For example, the large form of P-TEFb is disrupted by treatment of cells with P-TEFb inhibitors, UV light or actinomycin D (15), due to a poorly understood feedback loop between inhibition of transcription elongation and P-TEFb activity. Two physiological models for regulation of P-TEFb activity by 7SK and HEXIM1 have been identified thus far. Cardiac hypertrophy has been shown to be a consequence of activation of P-TEFb in cardiomyocytes through release from the large complex, which results in an increase in RNA polymerase II phosphorylation, gene expression and cell size (27). Conversely, HEXIM1 has been identified as a suppressor of breast cancer (28,29).

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The human immunodeficiency virus (HIV) replicates its genome by utilizing the host cell RNA polymerase II transcription machinery and is controlled mainly at the level of elongation. Basal transcription from the HIV long terminal repeat (LTR) promoter is extremely inefficient, with most RNA polymerase II initiation events terminating in abortive elongation (30). Transcription from the LTR is activated by the viral protein Tat, which facilitates the transition from abortive to productive elongation (31) by recruiting P-TEFb (4,32,33). Tat associates with the transactivation response element, TAR, which is an RNA stem-loop present in the nascent transcript produced from the HIV LTR, and P-TEFb containing cyclin T1 is recruited through association with both Tat and TAR (33–35). Interestingly, all P-TEFb inhibitors found to date block HIV replication at lower concentrations than those that have a negative effect on cellular transcription or viability (32,36,37). Additionally, over-expression of a kinase dead Cdk9 mutant reduced the expression of endogenous Cdk9 and inhibited HIV replication without affecting cell survival (32,36), and a partial knockdown of Cdk9 and cyclin T1 did not affect cell viability, yet inhibited Tat transactivation in Magi cells (38). While the mechanism of this heightened requirement for P-TEFb activity in Tat transactivation compared to normal cellular transcription is not understood, it is possible that much higher P-TEFb activity levels are required for HIV to overcome the tight repression of the HIV LTR promoter. In support of this, it has been shown that for HIV to replicate in primary blood lymphocytes (PBLs), the cells must be activated, which causes an increase in cyclin T1 expression and overall P-TEFb activity (39). Notably, much of the work regarding the role of P-TEFb in Tat transactivation was carried out prior to work on P-TEFb control and the discovery of the large form of P-TEFb, and its role in HIV replication has only begun to be explored.

Several lines of evidence led us and others (40–42) to hypothesize that the large form of P-TEFb is involved in HIV replication. It has been reported that activation of PBLs, which leads to increased P-TEFb as discussed above, causes an increase in 7SK levels along with the increase in P-TEFb expression, suggesting that activation of PBLs actually results in an increase in the large form of P-TEFb (40). The partial knockdown of Cdk9 and cyclin T1 that blocked Tat transactivation in Magi cells also eliminated high salt-activated (large form) P-TEFb (38). Furthermore, the majority of P-TEFb within cells is actually sequestered within the large complex. Taken together with the P-TEFb inhibition studies demonstrating that HIV requires much higher levels of P-TEFb activity than cellular promoters, this suggests that the P-TEFb within the large complex is necessary for HIV replication, and furthermore, that HIV has a mechanism to subvert normal cellular control and activate P-TEFb. This activation would provide a larger pool of P-TEFb for use at the HIV LTR (32,36,37). In support of this hypothesis, low levels of actinomycin D, which inhibit transcription and lead to release of P-TEFb from the large form without inhibition of P-TEFb itself (15), have been shown to activate the HIV LTR in the absence of Tat (43),

suggesting that the HIV LTR may be able to use the P-TEFb obtained from the large complex. Our current studies were designed to uncover details of how HIV interfaces with the cellular factors that control P-TEFb.

MATERIALS AND METHODS

Cell lines and compounds

All cells were grown under standard conditions of 37°C, 5% CO₂. HeLa37 cells, which express CD4 and both CXCR4 and CCR5 (44), were grown in DMEM with 10% FBS and 1 × penicillin/streptomycin (pen/strep). HeLa S3 cells were grown in DMEM-F12 with 10% FBS. 293T cells were obtained as a kind gift from P. McCray (Univ., Iowa). Cells were maintained in DMEM with 10% fetal calf serum and pen/strep. Cells were trypsinized and split 1:10 every 4 days.

Antibodies

Anti-Cdk9 (C-20) was obtained from Santa Cruz (sc-484). Anti-FLAG M2-peroxidase (HRP) was obtained from Sigma (A8592). Anti-cyclin T1 (T-18) used in western blots was obtained from Santa Cruz (sc-8127). Anti-cyclin T1 used in EMSA was generated in sheep recognizing the C-terminal domain (Abcam ab27963).

Generation of HIV p256 or VSVG pseudotyped p256 viral stocks

Here, 293T cells were seeded at 5×10^5 cells/well in a six-well tray a day before transfection with 7 µg of p256 proviral DNA or cotransfection with 6.5 µg of p256 proviral DNA and 0.5 µg of VSVG-expressing plasmid using the CaPO₄ procedure (45) to generate p256 and VSVG pseudotyped p256 viral stocks, respectively. Virus-containing supernatants were collected at 24, 48, 72 and 96 h post-transfection and virus production was measured by titrating the cell-free supernatants of p256 on HeLa37 cells and VSVG pseudotyped virions on HeLa S3 cells.

HIV infection

HeLa37 cells were infected with dual-tropic HIV p256 at an MOI of 0.1 for glycerol gradient analysis. After 4 days, the cells were lifted from the plate. A total of 4×10^4 cells were re-plated and immunostained for HIV antigens. The remaining cells were harvested and washed with 1 × PBS containing 1 µl/ml protease inhibitor cocktail (Sigma). Lysates were prepared to extract all P-TEFb and subjected to glycerol gradient sedimentation at 45 000 r.p.m. for 16 h as described below.

Transfection and Expression of HIV Tat

pFLAG-CMV2-Tat was created by cloning HIV-1 Tat (amino acids 1–86) in frame with the amino terminal FLAG-tag in pFLAG-CMV2 (Sigma) using EcoRI and BamHI restriction sites. Here, 293T cells were trypsinized and 3×10^6 cells were plated on a 150-mm plate. The next day when cells were ~80% confluent, the media was refreshed prior to calcium phosphate transfection. Seventy-five micrograms of plasmid DNA was added to

0.4 M CaCl₂ in 1 ml of 1 mM Tris (pH 7.6) and 0.1 mM EDTA. This tube was mixed well and maintained at 37°C until mixed in a dropwise fashion with 1 ml of 2× HBS (50 mM HEPES, 1.5 mM Na₂HPO₄ and 140 mM NaCl, pH 7.05) that was also held at 37°C. Mixture was vortexed thoroughly and added in a dropwise manner to the plate of cells. Media was refreshed at 12 h post transfection and maintained for an additional 30–36 h. At the termination of the transfection, a small population of the cells were immunostained for FLAG expression in a 48-well tray. Cells were fixed with 3.7% paraformaldehyde for 20 min followed by 3× 1 ml washes with PBS. Cells were permeabilized with 0.25% Triton X-100 in PBS for 20 min and washed extensively with PBS at the completion of the incubation. Cells were incubated with PBS containing 5% fetal calf serum (FCS) to block non-specific binding. Anti-FLAG monoclonal antibody M2 (Sigma) was diluted 1:1250 in PBS with 5% FCS and incubated with the cells for 2 h at 37°C. Cells were rinsed three times with PBS and peroxidase-conjugated goat anti-mouse antisera (1:1000) was added until the reaction was sufficiently developed. The remaining cells were harvested and washed with 1× PBS, and cell lysates were prepared to extract all P-TEFb and subjected to glycerol gradient sedimentation at 45 000 r.p.m. for 16 h as described below.

Glycerol gradient sedimentation analysis of cell lysates

Cell lysates were prepared in Buffer A (10 mM KCl, 10 mM MgCl₂, 10 mM HEPES, 1 mM EDTA, 1 mM DTT, 0.1% PMSF and EDTA-free complete protease inhibitor cocktail from Roche) containing 150 mM NaCl and 0.5% NP-40 as previously described (15,21). The lysates were clarified by centrifugation at 14 000 r.p.m. for 10 min at 4°C in a microfuge. The supernatant was used as input for 4.8 ml, 5–45% glycerol gradients, in Buffer A with 150 mM NaCl, spun at 45 000 r.p.m. for 20 or 16 h in a SW-55Ti rotor in a L7-55 Beckman ultracentrifuge as previously described (15,21). The fractions were analyzed by western blotting with anti-cyclin T1 or anti-Cdk9 for P-TEFb, and a HRP-conjugated M2 FLAG antibody for FLAG-Tat. Following incubation with the appropriate HRP-conjugated secondary antibody, when necessary, the blots were treated with Super Signal Dura West (Pierce). The western blots were imaged with a cooled CCD camera (UVP) and the P-TEFb signals in the fractions containing the free and large forms of P-TEFb, as indicated in the figures, were quantitated with Lab Works 4.0.

Kinase assay

Kinase reactions were carried out with recombinant purified P-TEFb [Cdk9/cyclin T1 (1–290)] and DSIF in 30 mM KCl, 20 mM HEPES pH 7.6, 7 mM MgCl₂, 30 μM ATP, 2.5 μCi of [γ -³²P]-ATP (Perkin-Elmer) with 1 μg BSA. The reactions were incubated for 20 min at 30°C and stopped by the addition of SDS-PAGE loading buffer. Reactions were resolved by SDS-PAGE on a 7.5% polyacrylamide gel. The dried gel was subjected to autoradiography and quantitation was performed using an InstantImager (Packard).

Equilibrium electrophoretic mobility shift assay (EMSA)

Twelve-microliter reactions were carried out in 25 mM HEPES, pH 7.6, 15% glycerol, 60 mM KCl, 10 mM DTT, 0.01% NP-40, 1 μg BSA with 200 ng yeast tRNA (Invitrogen) with or without 15 μM ZnCl₂ as indicated, and including T7 transcribed, radiolabeled 7SK, recombinant Tat (NIH AIDS reagents program), HEXIM1, and P-TEFb containing cyclin T1 as indicated. Cold RNA oligos for competition studies were chemically synthesized (IDT). 7SK and tRNA were heated for 5 min at 75°C and cooled on ice for another 5 min, prior to addition. Reactions were incubated at room temperature for 20 min and resolved on a 4% polyacrylamide gel in 0.5× Tris/glycine at room temperature for 2 h at 4 W. The dried gel was subjected to autoradiography.

Stoichiometric EMSA

Twelve-microliter reactions were carried out in 25 mM HEPES, pH 7.6, 15% glycerol, 60 mM KCl, 15 μM ZnCl₂, 5 mM DTT and 0.01% NP-40, including 50 ng of chemically synthesized TAR RNA (nucleotides 1–59) (Dharmacon), 640 c.p.m. of T7-transcribed radiolabeled TAR RNA, HEXIM1 and P-TEFb containing cyclin T1 (residues 1–290), as indicated. TAR RNA was heated for 5 min at 75°C and cooled on ice for another 5 min, prior to addition. Reactions were incubated at room temperature for 10 min and resolved on a native 5% polyacrylamide gel in 0.5× Tris/glycine at 4°C for 1.5 h at 4 W. The dried gel was subjected to autoradiography.

RESULTS

The large form of P-TEFb is reduced during HIV infection

Because most P-TEFb is found in the large inactive complex, and because several studies suggested that HIV requires higher P-TEFb activity than cellular promoters, we decided to test the hypothesis that HIV is able to release P-TEFb from the large form. First, the effect of HIV on the large form of P-TEFb was investigated through examination of the relative levels of the free and large forms of P-TEFb in HIV infected cells. HeLa37 cells were infected with the dual tropic HIV virus p256 (46) at an MOI of 0.1. Four days post-infection, the HeLa37 cells were determined to be ~96% positive for the production of viral proteins by immunohistochemistry. The cells were lysed to extract all of the P-TEFb and the forms of P-TEFb were analyzed by glycerol gradient sedimentation and quantitative western blotting (Figure 1A). Qualitatively, there is an obvious shift of both cyclin T1 and Cdk9 from the large to the small form following HIV infection. The Cdk9 signals for the small and large forms of P-TEFb from two experiments were averaged and SDs calculated (Figure 1B) to reveal that 84% ± 1% of P-TEFb was found in the large form in untreated cells, whereas after 4 days of HIV infection, 72% ± 3% of P-TEFb remained in the large form. The change in the relative level of the large form of P-TEFb by HIV infection suggests that HIV is able to release and utilize P-TEFb obtained from the large form.

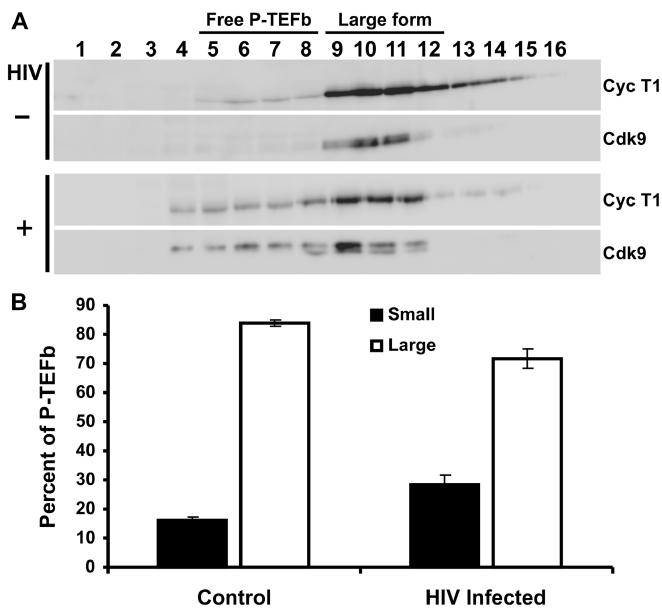


Figure 1. HIV infection leads to activation of P-TEFb. (A) HeLa37 cells were infected with p256 HIV at an MOI of 0.1. After 4 days, the lysates of HIV infected HeLa37 cells and a parallel culture of uninfected HeLa37 cells were lysed to extract all P-TEFb and subjected to glycerol gradient sedimentation. The fractions were examined by quantitative western blotting for Cdk9 and cyclin T1 as indicated. (B) Cdk9 was quantitated for small and large forms of P-TEFb, in either uninfected or infected HeLa37 cells, from duplicate experiments and averages were calculated and plotted. Error bars represent ± 1 SD of the mean.

Expression of HIV Tat leads to release of P-TEFb from the large form *in vivo*

Because the main difference between the activation of normal cellular promoters and the HIV LTR is the requirement for Tat and it is known that Tat is able to interact with P-TEFb, we hypothesized that the shift from large to free form observed during HIV infection was due to the expression of Tat in the HIV infected cells. To investigate this possibility, the effect of Tat on the large and small forms of P-TEFb present in uninfected cells was examined. In two separate experiments, 293T cells were transiently transfected with a construct expressing an N-terminally FLAG-tagged Tat. After 48 h, the cells were lysed and the P-TEFb was analyzed by glycerol gradient sedimentation and fractionation followed by quantitative western blotting. In control untransfected or β -Gal transfected 293T cells, most of the P-TEFb ($\sim 90\%$) is found in the large, inactive form [Figure 2A (fractions 11–15) and B (fractions 9–12)]. In cells expressing HIV Tat, there was a significant shift in P-TEFb from the large, inactive form to fractions that normally contain the free form [Figure 2A (fractions 6–10) and B (fractions 5–8)]. Quantitation of the amounts of Cdk9 indicated that in the first experiment, expression of Tat caused a reduction of the large form from 90 to 56%. In the second experiment, the large form was reduced from untransfected control and β -Gal control (89 and 81%, respectively) to 36%. The transfection efficiency was not the same in the two experiments and, interestingly, the fractional change in the

large form of P-TEFb correlated with the fraction of cells observed by immunostaining to be expressing Tat. This suggests that all of the P-TEFb was released from the large form within the transfected cells and that the remaining large form detected on the gradient was derived from untransfected cells. The larger change seen in these experiments compared to the experiment in which cells were infected with HIV is most likely due to the fact that Tat is present in higher amounts after transient transfection with a plasmid expressing Tat from the CMV promoter compared to the expression of Tat during HIV infection.

In cells expressing HIV Tat, the glycerol gradient fractions containing the ‘free’ form of P-TEFb appear to be shifted slightly down the gradient compared to untransfected or β -Gal transfected cells, and Tat, with a molecular weight of 15 kDa, appears to co-sediment with the small form of P-TEFb, a complex with a molecular weight of at least 120 kDa (Figure 2A and B). These observations suggest that Tat is bound to P-TEFb, and, in fact, co-immunoprecipitation of P-TEFb and HIV Tat from these fractions confirmed this interaction (data not shown). This association is consistent with many previous studies demonstrating Tat association with P-TEFb (4,39,47,48). Although an interaction with Brd4 and P-TEFb has been recently described (49–51) we do not think that expression of Tat in cells leads to the formation of a P-TEFb–Brd4 complex. The P-TEFb in fractions 4–8 of the glycerol gradients does not contain Brd4 because the 150 mM salt conditions used to extract P-TEFb from the nucleus do not extract Brd4. The interaction between Brd4 and P-TEFb can only be observed if nuclei are extracted with high salt (>300 mM) and then dialyzed down to low salt (50,51). From these studies we conclude that expression of Tat leads to the disruption of the large form and the formation of a Tat–P-TEFb complex.

HIV Tat binds to 7SK, competes with HEXIM1 and disrupts the large form of P-TEFb *in vitro*

The *in vivo* studies just presented indicate that the expression of Tat leads to the release of P-TEFb from the P-TEFb–HEXIM1–7SK complex and to the formation of a P-TEFb complex. However, due to the ambiguity of the *in vivo* system, it is not clear whether this release is due to a direct effect of Tat on the complex or more a more indirect effect, such as Tat association with P-TEFb complex leading to lower levels of P-TEFb activity and less general transcription that might trigger the cellular mechanism that releases P-TEFb from the large form. Because of this, a defined *in vitro* system was used to investigate the biochemical mechanism of this release. EMSAs were carried out under equilibrium binding conditions with protein in vast excess over 32 P-labeled *in vitro* transcribed full-length 7SK. Two-hundred nanograms of highly structured tRNA (>1000 -fold excess over 7SK) was used in each reaction as a non-specific competitor instead of poly (rI):poly (rC) that was used previously (19,21,25,52) because we recently found that HEXIM1 binds tightly to dsRNA (53). Dimeric HEXIM1 (25) formed its characteristic specific complex with 7SK

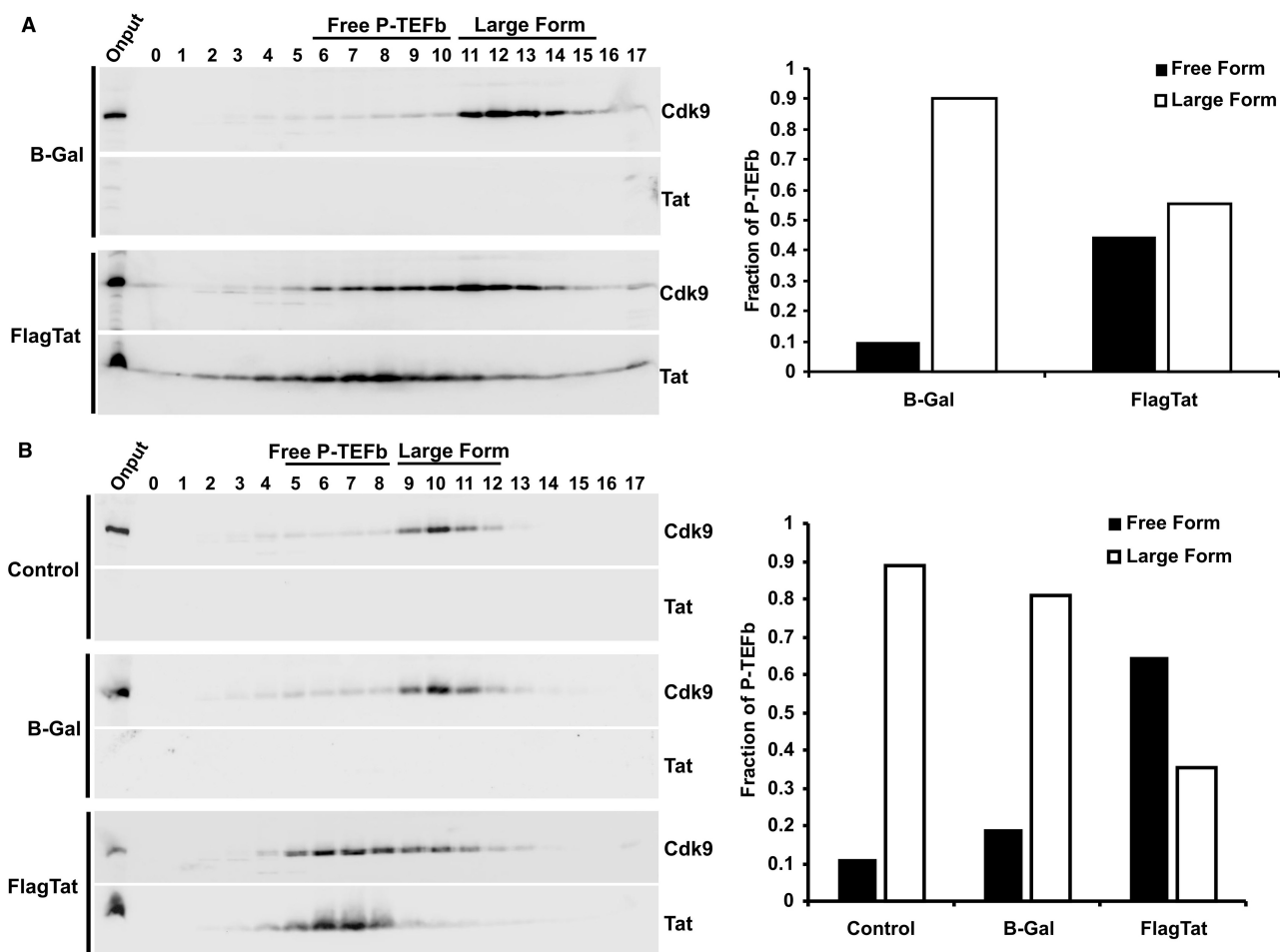


Figure 2. HIV Tat releases P-TEFb from the large form *in vivo*. (A) 293T cells were transiently transfected with a HIV Tat-expressing construct containing a N-terminal FLAG tag. After 48 h, control 293T cells (β -Gal-transfected) and FLAG-Tat transfected 293T cells were lysed to extract all P-TEFb and subjected to glycerol gradient sedimentation. The gradient fractions were analyzed by quantitative western blotting for Cdk9 or FLAG-Tat, as indicated. (B) Repeat experiment, as in (A), with an additional mock transfection control (Control) and with higher transfection efficiencies.

(Figure 3A, H1-7SK). Somewhat surprisingly, as increasing amounts of Tat were added to 7SK a more slowly migrating complex formed, demonstrating that Tat can bind to 7SK forming a Tat-7SK complex. A portion of the 7SK was also shifted into the well and this could be due to association of 7SK with oxidized aggregates of Tat. When HEXIM1 was pre-incubated with 7SK to form the HEXIM1-7SK complex and then increasing amounts of Tat were added before being loaded onto the native gel, the HEXIM1-7SK complex was gradually eliminated and was replaced by a Tat-7SK complex. When the Tat-7SK complex was preformed and then HEXIM1 was added, the competition was even stronger. The amounts of the Tat-7SK complex formed were virtually identical with or without HEXIM1 being present. The fact that larger molar amounts of Tat were required is likely due to the presence of a large fraction of inactive Tat in the preparation used. We conclude that Tat can compete strongly with HEXIM1 for binding to 7SK, and that even when the relatively stable HEXIM1-7SK complex is preformed, Tat can disrupt it. There was no evidence for a significant amount of a Tat-HEXIM1-7SK complex,

indicating that Tat and HEXIM1 bind to an identical or overlapping region of 7SK or that Tat and HEXIM1 bind to different mutually exclusive conformations of 7SK.

We next examined the effect of Tat on the P-TEFb-HEXIM1-7SK complex. In control reactions, the previously observed HEXIM1-7SK and Tat-7SK complexes were found. P-TEFb, containing Cdk9 and full-length cyclinT1, formed non-discrete complexes with 7SK (Figure 3B). This is likely due to a weak, perhaps non-specific, interaction of P-TEFb and 7SK. A combination of Tat and increasing amounts of P-TEFb also gave a progression of non-specific shifts. However, when HEXIM1 and increasing amounts of P-TEFb were added together, the HEXIM1 shift gradually disappeared and a very discrete band containing HEXIM1 and P-TEFb formed (Figure 3B, P-H1-7SK). To investigate the direct effect of Tat on formation of a P-TEFb-HEXIM1-7SK complex, HEXIM1, P-TEFb and increasing amounts of HIV Tat were pre-incubated followed by the addition of 7SK. As Tat was titrated into the reactions, a dose-dependent inhibition of the formation of the P-TEFb-HEXIM1-7SK complex resulted with the

To further examine Tat inhibition of P-TEFb-HEXIM1-7SK complex formation and to determine if Tat could disrupt a preformed complex, EMSA was again used (Figure 4B). Again, Tat was observed to inhibit the formation of the P-TEFb-HEXIM1-7SK complex (Figure 4B, Inhibition). Additionally, as more P-TEFb was added to a constant amount of HEXIM1 in the absence of Tat (compare the first lane in each set of P-TEFb levels), there was the expected progressive shift of the HEXIM1-7SK complex to the P-TEFb-HEXIM1-7SK complex. As increasing amounts of Tat were included in the reactions, there was a progressive decrease in the P-TEFb-HEXIM1-7SK complex formed and corresponding increases in the HEXIM1-7SK complex and the previously postulated Tat-P-TEFb-7SK complexes. Interestingly, as the amount of P-TEFb was increased in the reactions, there was a progressive shift from the lower Tat-P-TEFb-7SK band to the upper band (compare the last lane of each set of P-TEFb), supporting the hypothesis that the upper band is a Tat-P-TEFb-7SK complex containing two molecules of P-TEFb. Again, only at the highest concentrations of Tat, after all P-TEFb has been bound by Tat, was the Tat-7SK complex observed. To determine if Tat is able to disrupt a preformed large complex, P-TEFb-HEXIM1-7SK complexes were formed before addition of Tat. As more Tat was added to preformed P-TEFb-HEXIM1-7SK complexes, there was a disruption of the complex, with formation of HEXIM1-7SK, Tat-P-TEFb-7SK, but not the Tat-7SK complexes (Figure 4B, Disruption). Disruption of pre-formed P-TEFb-HEXIM1-7SK complexes by Tat appeared less efficient than inhibition of complex formation when the amount of P-TEFb-HEXIM1-7SK remaining at corresponding amounts of Tat is compared between the two experiments. Overall, these data demonstrate that Tat can prevent the formation of a P-TEFb-HEXIM1-7SK complex and can disrupt a preformed P-TEFb-HEXIM1-7SK complex.

Tat binds to nucleotides 10-48 of 7SK

To gain insight into what sequence Tat recognized in 7SK, the first 100 nt of 7SK were compared to HIV TAR, and a set of oligos were designed and tested for their ability to block Tat binding to 7SK. The target for Tat binding in TAR consists of an AUCUG forming a bulge in the apical region of the TAR stem-loop and this binding of Tat changes the structure of TAR so that a pocket forms between U23 and G26 (54-56). When the sequence of 7SK was analyzed, three AUCUG motifs were found (Figure 5A). This sequence would be expected to occur randomly once every 4⁵ bp, or about 1 in 1000 nt, and so it may be significant that it is found three times in the first 100 nt of 7SK. A set of oligos were designed that contained AUCUG sequences in the context of sequences that would be unstructured or that could form secondary structure with the AUCUG in loops or in bulges. Other regions of 7SK were also sampled as controls (Figure 5A). mFold suggested that 7SK (10-48) would form a stem-loop with one AUCUG in a bulge and one in the loop and is shown compared to TAR in Figure 5B. HEXIM1 has

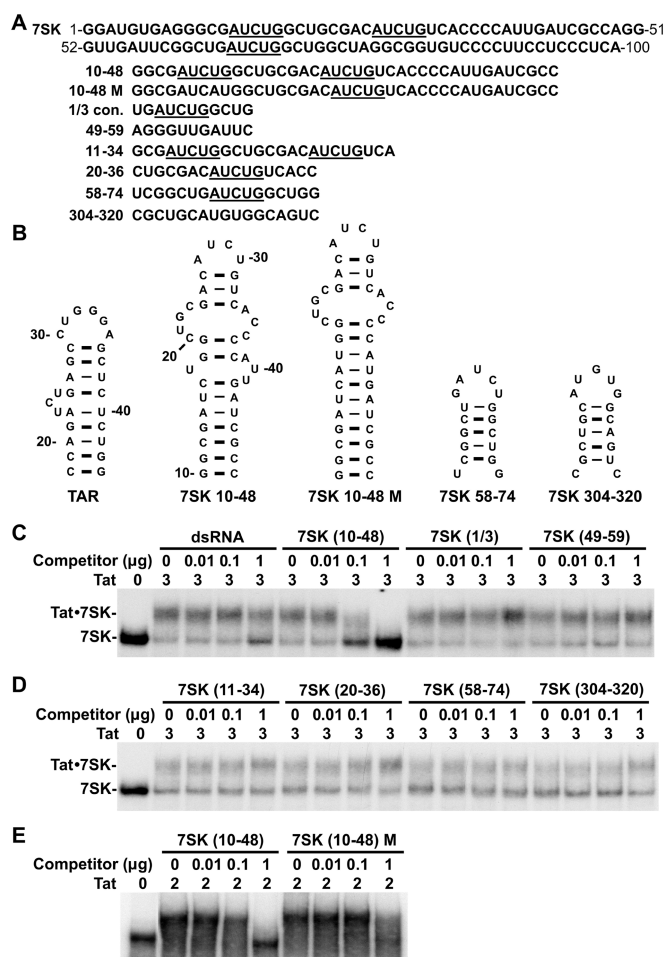


Figure 5. HIV Tat binds to a region of 7SK resembling TAR. (A) Analysis of the sequence of 7SK reveals three AUCUG Tat consensus-binding sites in the first 100 nt. Several structured and unstructured RNA oligos with or without this consensus sequence were designed and chemically synthesized with most coming from the native 7SK sequences indicated. 7SK (10-48 M) has one insertion and one deletion in an otherwise wild-type 7SK (10-48) sequence. (B) Predicted structures of all oligos with sufficient stability to be the predominate form at room temperature. (C) Competition EMSA analysis of Tat-7SK complex formation. Tat, ³²P-labeled 7SK, and the indicated cold RNA oligos were pre-incubated and the resulting complexes were resolved by gel electrophoresis on a native gel, followed by autoradiography to visualize the 7SK shift. The dsRNA was a 25-bp double-stranded RNA unrelated to 7SK sequence described previously (53). (D) Competition EMSA comparison between 7SK (10-48) and 7SK (10-48 M). Note in this experiment the 7SK was of higher specific activity than in (C) so less Tat was needed to achieve a higher fraction of Tat-7SK complex.

been demonstrated to bind to a region of in the 5' end of 7SK (57) and to this specific oligo as well as it binds to intact 7SK (53). Unlabeled oligos were then used as competitors in EMSAs with labeled full-length 7SK and Tat. No competition was seen for any of the oligos except for 7SK (10-48) (Figure 5C and D). This strongly suggests that Tat associated with the residues from 10 to 48 of 7SK. Because no competition was seen with oligos comprised of 11-34 or 20-36, Tat may require the secondary structure shown in Figure 5B to be able to interact with 7SK.

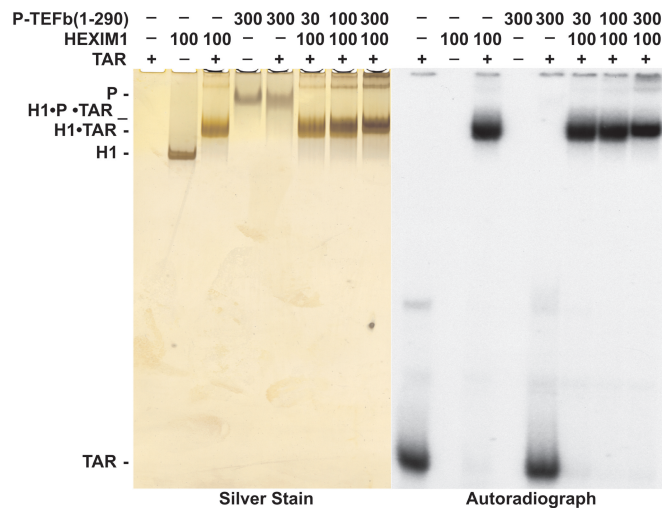


Figure 6. HEXIM1 binds TAR and the HEXIM1-TAR complex recruits P-TEFb. The binding of HEXIM1, TAR and P-TEFb was evaluated under stoichiometric conditions by EMSA. The indicated components were pre-incubated and the resulting complexes were resolved by gel electrophoresis on a native gel. The gel was silver-stained to visualize protein shifts and autoradiography used to visualize the ^{32}P -labeled RNA shifts.

To further analyze the specificity of the interaction of Tat with 7SK, a new oligo similar to 7SK (10–48) was used in a competition EMSA assay. The mutant oligo, 7SK (10–48 M), was identical to 7SK (10–48) except that an A was inserted in the first AUCUG site (AUCAUG) and U40 was deleted (Figure 5A). These two changes eliminated the bulge region to which we thought Tat might bind and increased the length of the stem (Figure 5B). The mutant oligo serves as a control for the potential binding of Tat to any RNA that contains a stem and loop with a bulge. As was seen above, as increasing amounts of the 7SK (10–48) were included in reactions containing Tat and ^{32}P -7SK, the Tat-7SK shift was decreased with 100 ng of the oligo and completely eliminated with 1000 ng of the oligo (Figure 5E). In contrast, 7SK (10–48 M) only partially blocked Tat binding to 7SK at the highest level of oligo. We conclude that the changes in the 7SK 10–48 oligo lowered the affinity of Tat. Our results up to this point indicate that Tat binds to 7SK in a specific manner and that the region of 7SK to which Tat binds is from nucleotides 10–48.

HEXIM1 binds to TAR RNA and inhibits P-TEFb

Given the previous results demonstrating that HEXIM1 and Tat compete for binding to the same small 7SK oligo, and the similarity of this region with the TAR RNA, we explored the possibility that HEXIM1 might bind to TAR. If this is the case, it might also be able to bind and inhibit P-TEFb much the same way that HEXIM1 and 7SK inhibit P-TEFb, thus repressing P-TEFb, and transcription elongation, at the HIV LTR promoter. To begin testing this hypothesis, EMSAs were used to determine if HEXIM1 could bind to TAR and recruit P-TEFb. ^{32}P -labeled, chemically synthesized TAR RNA was combined with FPLC-purified HEXIM1,

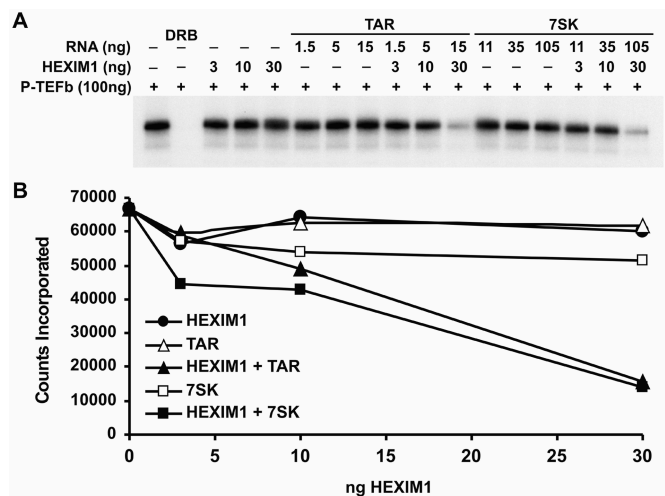


Figure 7. HEXIM1-TAR inhibits the kinase activity of P-TEFb. (A) HEXIM1, TAR RNA, 7SK RNA or mixtures of HEXIM1 and TAR or 7SK found by EMSA to result in 1:1 HEXIM1-TAR or HEXIM1-7SK complexes were titrated into *in vitro* kinase assays containing purified P-TEFb and incorporation of ^{32}P into the Spt5 subunit of DSIF was analyzed by SDS-PAGE followed by autoradiography. (B) Kinase activity of P-TEFb in the presence HEXIM1, TAR and 7SK as described in (A) was quantitated using an Instant Imager and plotted as a function of increasing HEXIM1.

and P-TEFb containing Cdk9 and the first 290 amino acids of cyclin T1, as indicated; complexes were resolved on a native gel and both silver-stained and exposed to film to visualize both the RNA and protein shifts (Figure 6). When HEXIM1 and TAR were combined, a new complex migrating more slowly than either HEXIM1 or TAR RNA was observed, suggesting the formation of a HEXIM1-TAR complex (Figure 6, H1-TAR). As P-TEFb was titrated onto this complex, there was a further shift of the complex with more mobility than free P-TEFb, accompanied by a change in the color of the silver-stained gel complex, suggesting the addition of a new component and the formation of a P-TEFb-HEXIM1-TAR complex (Figure 6, H1-P-TAR). More importantly, free P-TEFb, which does not bind to RNA alone, disappeared.

While this result supports the hypothesis that HEXIM1 can bind to TAR, with subsequent recruitment and binding of P-TEFb, inhibition of P-TEFb requires that the RNA change the conformation of HEXIM1 to relieve the autoinhibitory properties of the protein. To determine whether HEXIM1-TAR can inhibit P-TEFb, *in vitro* kinase assays were performed using recombinant P-TEFb(1–290), HEXIM1 and TAR RNA as in the EMSAs to monitor γ - ^{32}P (ATP) incorporation into DSIF, and the complexes resolved by SDS-PAGE (Figure 7A). Compared to P-TEFb alone and with DRB, an inhibitor of P-TEFb, there was no qualitative effect of HEXIM1 alone on P-TEFb kinase activity. Similarly, TAR alone, or 7SK alone as a control, demonstrated no appreciable effect. However, when HEXIM1 and TAR are combined and titrated in a constant ratio determined by EMSA to give 1:1 HEXIM1-TAR binding, there was a dose-dependent inhibition of P-TEFb, similar to that observed

with HEXIM1–7SK. When the assay was quantitated (Figure 7B), HEXIM1 and the RNAs separately had no significant effect; however, HEXIM1–TAR appeared to inhibit as effectively as equimolar amounts of HEXIM1–7SK. These findings support the hypothesis that HEXIM1 may actually be inhibiting P-TEFb at the HIV LTR.

DISCUSSION

The initial goal of this work was to examine the role of P-TEFb in the large form in HIV replication. In the course of our studies a number of important findings were made that can be applied toward understanding how P-TEFb is regulated and how the P-TEFb regulatory machinery may be used during HIV replication. Importantly, we found that the large form of P-TEFb was reduced during viral infection of HeLa37 cells. Furthermore, expression of HIV Tat in 293T cells was sufficient for release of P-TEFb from the large form. *In vitro* binding studies indicated that Tat could bind to 7SK and could block the formation of the P-TEFb–HEXIM1–7SK complex, and could remove P-TEFb from a preformed large complex. Finally, we found that HEXIM1 could bind to HIV TAR, and recruit and inhibit P-TEFb *in vitro*.

HIV Tat activates the large form of P-TEFb

The results presented in this study strongly suggest that the large form of P-TEFb is involved in the positive regulation of HIV replication. Interestingly, upon infection with HIV, there was a decrease in the large form of P-TEFb, and we subsequently demonstrated that the HIV Tat protein in the absence of other viral proteins or RNAs could release P-TEFb from the large form, with the formation of a Tat–P-TEFb complex. Potentially relevant to this, actinomycin D, which globally inhibits transcription, has been shown to cause release of P-TEFb from the large form and can also activate transcription from the HIV LTR in the absence of Tat (43). Presumably, this is due to increased P-TEFb activity. This data suggests that HIV replication does not require the large form *per se*, but rather requires higher levels of P-TEFb activity than cellular promoters to overcome the inherent negativity of the HIV LTR. Because the vast majority of P-TEFb is within the large form, HIV may have developed a mechanism to increase P-TEFb activity required for productive elongation from the HIV LTR via Tat-mediated release from the large complex. However, increasing P-TEFb activity has deleterious effects within the cell, as it has been shown that excess P-TEFb in cardiomyocytes leads to apoptosis (58) and disequilibrium between the large and free forms of P-TEFb due to exogenous expression of P-TEFb subunits results in degradation of endogenous P-TEFb in order to maintain constant P-TEFb levels (59,60). In using Tat, HIV is not only using its genome efficiently, as Tat is the factor that ultimately recruits P-TEFb to the LTR, it may also be masking the increase in P-TEFb activity by not releasing ‘free’ P-TEFb, but instead forming a Tat–P-TEFb complex. This complex might not be able to function at cellular promoters because of its inability to interact with

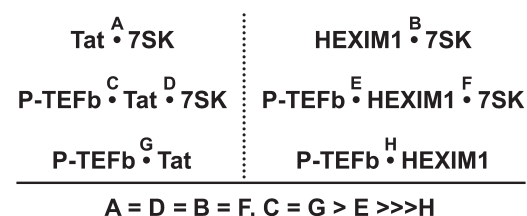


Figure 8. Relative interaction potentials. All complexes examined in this study are indicated and the various protein–protein and protein–RNA interactions are lettered. As described in the text, two hierarchies of interaction potentials are indicated.

other transcription factors, as evidenced by the ability of Tat to inhibit CIITA function (48).

While Tat can compete with HEXIM1 for binding to 7SK, it appears that the mechanism by which Tat is able to release and activate P-TEFb from the large form may be via direct interaction with P-TEFb and inhibition of the formation of the large complex and/or direct disruption of it. Figure 8 shows all the complexes examined in this study. Where possible, relative interaction potentials (labeled with letters) were estimated from the studies presented. We were not able to detect any influence of P-TEFb on the interaction between Tat and 7SK, so A and D are similar. Likewise P-TEFb did not seem to influence the interaction of HEXIM1 with 7SK so B and F are similar. Because Tat was able to compete well with HEXIM1 for binding to 7SK, A and D may be slightly stronger than B and F. It was not possible to determine the relative interaction potential between P-TEFb and Tat compared to the RNA binding proteins and 7SK so a second hierarchy was created. Because the interaction of Tat and P-TEFb was not affected by 7SK, C is similar to G. Most importantly, P-TEFb preferred to interact with Tat rather than the HEXIM1–7SK complex, so G is stronger than E. Finally one of the biggest differences between Tat and HEXIM1 is that HEXIM1, but not Tat, requires 7SK to evoke a conformational change necessary for interaction with P-TEFb. This means that E is much greater than H. While the relative interaction potentials help explain what was observed *in vitro* and are consistent with what we found in cells, the *in vivo* situation is complicated by the presence of other proteins that may interact with 7SK. HEXIM1 is not found in association with 7SK in the absence of P-TEFb even though it has high affinity for the RNA. It is possible that the Tat–7SK interaction is also eliminated *in vivo* by a similar mechanism and this would in turn favor the formation of the Tat–P-TEFb complex.

HEXIM1 repression of the HIV LTR promoter

As previously discussed, the high sensitivity of HIV transcription to P-TEFb inhibitors compared to cellular transcription suggests that HIV requires much higher P-TEFb activity to overcome the inherent repression of the HIV LTR. The basis of this negativity, however, remains controversial. Some have suggested that it is due to recruitment of NELF (61). Unfortunately, this may not adequately account for the differential repression observed

between cellular and viral promoters, as this factor is also present at cellular promoters. During the course of these studies, clues to the basis of the repression of the HIV LTR promoter were discovered which allow for an alternative model to be proposed. It was found that HEXIM1 can bind to the TAR RNA element that is normally found in the nascent transcript generated from the HIV LTR promoter. Furthermore, this HEXIM1–TAR complex was shown to be able to recruit and bind P-TEFb, effectively inhibiting P-TEFb in the same manner as HEXIM1–7SK. From this, we hypothesize that the recruitment of HEXIM1 by TAR leads to repression of the HIV LTR. Only when there are abnormally high levels of P-TEFb, such as in the presence of low levels of actinomycin D, or when Tat is present would productive elongation from the HIV LTR be allowed.

In examining the control of P-TEFb or HIV LTR function several groups inadvertently provided evidence for a direct effect of HEXIM1 on the expression of the HIV LTR. When HEXIM1 was overexpressed in cells it had a negative effect on basal expression from an HIV LTR reporter gene (17,20,22,62). This effect did not require a functional TAR region of the HIV 5' UTR because an LTR reporter containing a mutation in the UCU bulge that stops Tat transactivation gave the same result. Inhibition did require the RNA binding domain and the P-TEFb inhibitory domain of HEXIM1 (62). The authors hypothesized that the extra HEXIM1 exerted its effect through inhibition of P-TEFb in a 7SK-dependent manner; however we (21) and others (18,22,23) have shown that HEXIM1 is already in excess (2–5-fold) in most cell lines examined. Knockdown of HEXIM1 had the opposite effect on HIV LTR expression. Reduction of HEXIM1 increased the basal, Tat-independent expression from a transfected HIV LTR and this effect was greater than with constructs that did not contain early transcribed HIV sequences (17). Although the authors explained the increase in expression as a result of the increase in free P-TEFb, we (21) and others (22) have shown that knockdown of HEXIM1, even to levels lower than achieved in the study being discussed, does not lead to an increase in free P-TEFb. Instead, as HEXIM1 levels fall, the free HEXIM1 in the cell enters the large form, and when there is not enough HEXIM1 then HEXIM2 takes over (21,22). Unknown cellular mechanisms must tightly regulate amount of P-TEFb activity keeping it at a constant level. Therefore, the best explanation for the results from all these studies, given the new understanding that HEXIM1 binds to TAR, is that there is a direct effect mediated by HEXIM1 binding to the nascent HIV transcript.

Hernandez and Pessler (63) discovered this negative effect of the HIV LTR many years ago. They found that the first 60 bp of the transcribed region of the HIV LTR was an inducer of short transcripts (IST). Unfortunately, at the time of those findings, P-TEFb had not been discovered and mechanisms of RNA polymerase II elongation control were not understood. It now seems likely that the IST was a P-TEFb repressor and we now hypothesize that the mechanism was through HEXIM1 binding to the 5' UTR. There is nothing special about the

HIV promoter in this regard as other promoters driving the early transcribed region of HIV give rise to short transcripts and are Tat responsive if TAR is intact (63).

The proposed direct effect of HEXIM1 as a repressor of P-TEFb through interaction with the HIV 5' UTR may prove to be exploitable as a target for antiviral treatments. Latent infections by HIV are the primary reason that antiviral treatments do not eradicate the virus. If HEXIM1 can be displaced from the HIV 5' UTR, the virus might not be able to maintain its latency and would, therefore, be unable to hide from antiviral treatments. It may be possible that HEXIM1 release from the HIV 5' UTR and from 7SK may be controlled in the same way. If so, then treatment of cells with agents that cause HEXIM1 release would not only relieve HEXIM1-mediated repression of the HIV LTR, but would also lead to increased levels of P-TEFb that would also stimulate HIV transcription even before Tat is produced.

In support of our finding here, while our manuscript was in revision another group reported studies that demonstrated the ability of Tat to cause the release of P-TEFb from the P-TEFb–HEXIM1–7SK complex (64). Binding studies were used to demonstrate that an interaction between amino acids 255–359 of HEXIM1 and a GST fusion containing cyclin T1 (1–292) was disrupted by Tat and similar results were obtained *in vivo* using tagged proteins (64).

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