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Isolation and functional characterization of P-TEFb-associated factors that control general and HIV-1 transcriptional elongation

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ABSTRACT

Originally identified as a factor crucial for RNA polymerase (Pol) II transcriptional elongation of cellular genes, the P-TEFb kinase was subsequently shown to also serve as a specific host co-factor required for HIV-1 transcription. Recruited by either the bromodomain protein Brd4 to cellular promoters for general transcription or the HIV-1 Tat protein to the viral LTR for activated HIV-1 transcription, P-TEFb stimulates the processivity of Pol II through phosphorylating the C-terminal domain of Pol II and a pair of negative elongation factors, leading to the synthesis of full-length transcripts. However, abundant evidence indicates that P-TEFb does not act alone in the cell and that all of its known biological functions are likely mediated through the interactions with various regulators. Although a number of P-TEFb-associated factors have already been identified, there are likely more yet to be discovered. Given that P-TEFb plays an essential role in HIV-1 transcription, a major challenge facing the field is to identify all the P-TEFb-associated factors and determine how they may modulate Tat-transactivation and HIV-1 replication. Described here is a set of experimental procedures that have not only enabled us to isolate and identify several P-TEFb-associated factors, but also provided the means to characterize their biochemical functions in HIV-1 transcriptional control. In light of the recent demonstrations that transcriptional elongation plays a much more important role in controlling metazoan gene expression than previously thought, the techniques presented here will also be useful for analyzing Pol II elongation of cellular genes.

1. Introduction

Accumulating evidence has highlighted the importance of P-TEFb, a heterodimer consisting of CDK9 and its regulatory subunit Cyclin T1 (CycT1, or the minor forms T2 and K), in controlling the transcription of a vast array of eukaryotic protein-encoding genes [1,2]. Through phosphorylating the C-terminal domain (CTD) of RNA polymerase (Pol) II and negative elongation factors DSIF and NELF, P-TEFb promotes the transition of Pol II from abortive to productive elongation, leading to the synthesis of the full-length RNA transcripts [1–3]. Studies employing molecular genetic methods and pharmacological inhibitors have revealed diverse functions of P-TEFb in regulating cell proliferation, differentiation, apoptosis and stress-responses [1,4,5]. Dysregulation of P-TEFb is known to cause the disruption of normal embryonic development, malignant transformation, and cardiac hypertrophy [1,5,6].

P-TEFb is critical for the transcription of not only cellular genes, but also the integrated HIV-1 proviral genome [1]. Pol II transcribing the HIV-1 LTR has a strong tendency to stall and then terminate near the start site, resulting in the production of only short transcripts. To prevent Pol II from stalling, P-TEFb must be recruited to the HIV-1 LTR by the viral Tat protein and TAR RNA structure that is located at the 5′ end of all viral transcripts. The localized P-TEFb then phosphorylates the Pol II CTD, DSIF, and NELF to stimulate HIV-1 transcriptional elongation [1,7]. Besides P-TEFb, our recent data indicate that ELL2, another potent transcription elongation factor known to function through suppressing transient pausing of Pol II, is also recruited to the HIV-1 LTR by Tat in conjunction with a cellular transcription factor/co-activator AFF4 [8]. The ability of Tat to enable two different classes of elongation factors to cooperate and coordinate their actions on the same Pol II enzyme explains why Tat is such a powerful activator of HIV-1 transcription.

In the absence of Tat, most of nuclear P-TEFb exist in two distinct complexes [1]. A catalytically inactive complex termed 7SK snRNP, which also contains the 7SK snRNA and nuclear proteins HEXIM1 (and/or the minor HEXIM2) [9–11], LARP7 (also termed PIP7S) [12], and MePCE (also termed BCDIN3) [13], sequesters about 70–80% of P-TEFb in the nucleus. Within this complex, the 7SK snRNA serves as a molecular scaffold to coordinate the interactions among the various protein components [14,15]. While HEXIM1 (and/or HEXIM2) inhibits the P-TEFb kinase activity

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[9,11], LARP7 and MePCE cooperate to protect 7SK against exonucleolytic decay [14]. It is interesting to note that through directly displacing HEXIM1 from CycT1, Tat is able to capture P-TEFb from 7SK snRNP to form a transcriptionally active complex [16] that contains ELL2, AFF4, Tat, and P-TEFb and is required for activated HIV-1 transcription [8].

Besides 7SK snRNP, P-TEFb also exists in another complex together with the bromodomain protein Brd4, which recruits P-TEFb to chromatin templates (including the HIV-1 LTR in the absence of Tat) through interacting with acetylated histones and/or the Mediator complex [17,18]. Although the Brd4–P-TEFb interaction is required for general transcriptional elongation including basal HIV-1 transcription [17,18], it is incompatible with Tat-transactivation as Brd4 and Tat compete for binding to the same surface of P-TEFb [16]. Consistent with this observation, it has been shown that the overexpression of Brd4 or the P-TEFb-interacting domain in Brd4 inhibits HIV-1 transcription and replication [19].

Accumulating evidence indicates that through alternatively associating with its positive and negative regulators, P-TEFb is kept in a functional equilibrium. Many agents or conditions that globally impact cell growth have been shown to dynamically regulate this equilibrium to produce P-TEFb levels that can accommodate the changing demands of cellular gene expression and growth [1,20]. To enable a better understanding of the regulation of the P-TEFb functional equilibrium, we and others have recently shown that the Ca^{2+}-calmodulin-PP2B/PP1α and PI3K/Akt signaling pathways play an important role in the disruption of 7SK snRNP, release of P-TEFb and formation of the Brd4–P-TEFb complex in response to certain extracellular stimuli such as UV irradiation or short-term HMBA treatment [21,22].

The evidence obtained so far does not support the existence of free P-TEFb in the nucleus. All the known biological functions of P-TEFb are likely mediated through the interactions of P-TEFb with its various function-specific regulators. Although a number of P-TEFb-associated factors have already been identified, there are likely more yet to be discovered. For example, our preliminary data suggest the existence of additional yet to be identified factors in the Brd4–P-TEFb complex (data not shown). Furthermore, there are probably additional unknown factors existing in the ELL2/AFF4/Tat/P-TEFb-containing complex [8]. Given that P-TEFb plays an essential role in HIV-1 transcription, a major challenge facing the field is to identify all the P-TEFb-associated factors and determine how they may modulate Tat-transactivation, HIV-1 replication and latency. Described below is a set of experimental procedures that we have used in the past to successfully isolate and identify several P-TEFb-associated factors and investigate their functions in HIV-1 transcriptional elongation. These procedures can be easily adapted to allow the identification and characterization of additional P-TEFb-associated factors in the future.

2. Procedures

The successful identification of P-TEFb-associated factors and analysis of their effects on HIV-1 transcription require materials in a highly purified and active state. Many affinity-purification strategies exist for the isolation of native or recombinant proteins. We favor the system involving tagging with the Flag epitope and immunoprecipitation using the anti-Flag M2 affinity gel (Sigma, Cat. No.: A2220). Unlike some of the bulky protein tags used in procedures such as the tandem affinity-purification (TAP) tagging [13,23], the Flag peptide sequence (DYKDDDDK) is short and highly hydrophilic and rarely interferes with the biochemical functions of the tagged protein. As a result, the tagged proteins are often completely indistinguishable from their endogenous counterparts in terms of biological activity and the Flag-tag is easily accessible by the anti-Flag monoclonal antibody. Finally, the ability to competitively elute the tagged protein and its associated factors off the affinity gel by using a buffer containing the synthetic Flag peptide (Sigma; Cat, No.: F3290) offers an additional level of purification and permits the eluted proteins to exist under mild salt and neutral pH conditions (Fig. 1).

2.1. Large-scale affinity-purification of P-TEFb and its associated factors

Given the observations that transiently overexpressed proteins often have artificial interactions with irrelevant proteins (data not shown), we routinely perform large-scale affinity-purification from nuclear extracts (NE) of an engineered HeLa-based cell line (called F1C2), which stably expresses the Flag-tagged CDK9-F at about the same level as the endogenous protein (Fig. 1). The procedure for generating F1C2 has been described previously [24]. The preparation of NE from F1C2 cells (in 80% confluence) follows the classic procedure by Dignam et al. [25] without any further dialysis (the final salt concentration is approximately 0.35 M NaCl). The purification of P-TEFb-containing complexes follows the protocol described below.

(A) Large-scale immuno-affinity-purification

(1) Setup pre-clearing and affinity-purification tubes containing Protein A (GE, Cat. No.: 17-5280-05) and anti-Flag beads (Sigma, Cat. No.: A2220), respectively. Wash beads three times with buffer D0.3 and then remove all traces of liquid by using a long-needled syringe such as a Hamilton 725N 250ul SYR (22s/2″/2) with the tip of the needle positioned at the very bottom of the packed beads.

(2) Pre-clear the NE by incubating with the pre-washed Protein A beads at a ratio of 1 mL of NE to 40 μL of packed beads for 30 min at 4 °C on a rotator.

(3) Spin at 2000*g* for 1 min. Transfer the supernatant (pre-cleared NE) to the tubes containing the pre-washed anti-Flag beads at a ratio of 1 mL of NE to 40 μL of packed beads. The incubation proceeds for 2 h at 4 °C on a rotator.

(4) Spin beads to the bottom of the tube, and then remove and discard the supernatant. Wash beads three times with buffer D0.3 and then twice with buffer D0.1. If two or more centrifuge tubes are used for one sample, combine beads together into one 1.5 mL Eppendorf tube at this stage.

(5) Remove all traces of liquid with a Hamilton syringe. Elute proteins off the beads by incubating with buffer D0.1 M containing 0.5 mg/mL of the Flag peptide (Sigma, Cat. No.: F3290) for 30 min at room temperature with occasional brief vortexing. Use 20 μL of the peptide solution for every mL of the starting NE.

(6) Spray the outside of the Eppendorf tube with 70% ethanol and wipe clean with a Kimwipe tissue. Next, a 25G5/8″ needle is used to FIRST poke a small hole in the lid of the tube to release the pressure inside and THEN a very small hole through the pointed end at the bottom of the tube. To make sure that the hole is just big enough to allow the liquid but not any beads, which can trap many non-specific proteins despite extensive washes, to pass through, the beveled needle tip should be inserted only halfway into the pointed end of the tube (Fig. 2).

(7) Insert the pointed end of the above tube that contains the beads and eluted proteins into a new 1.5 mL Eppendorf tube (Fig. 2). Put the two-tube set into a 50 mL centrifuge tube and spin at 1000*g* for 2 min to let the liquid containing the eluted proteins pass through the small hole and into the new Eppendorf tube (*note:* if small amounts of the anti-Flag beads are suspected to pass through the hole and end up in the final eluted protein sample, repeat steps 6 and 7 to remove all the beads). The affinity-purified CDK9-F and its associated factors are now ready for subsequent analyses by SDS–PAGE followed by silver-staining, Western blotting, mass spectrometry, and key functional tests (e.g. an in vitro

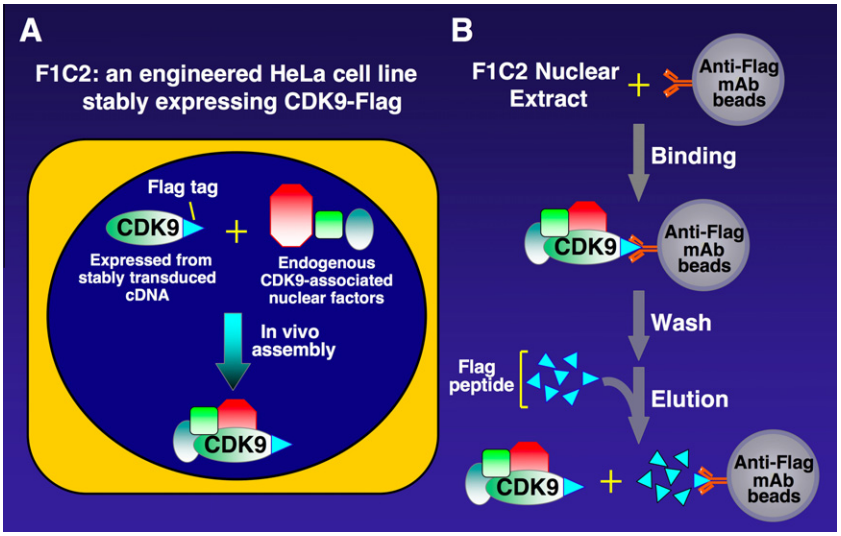

Fig. 1. Major steps involved in the affinity-purification of CDK9-associated factors. (A) The HeLa-based F1C2 cell line that stably expresses CDK9-Flag (CDK9-F) from a transduced retroviral vector has been established. CDK9-F interacts with its natural partners to form a P-TEFb-containing complex(es) in the nucleus. (B) Nuclear extracts (NE) prepared from F1C2 cells are incubated with the anti-Flag monoclonal antibody (mAb) beads, which specifically attract CDK9-F and its associated factors. After extensive washes, CDK9-F and its associated factors are eluted off the beads with a buffer containing synthetic Flag peptide.

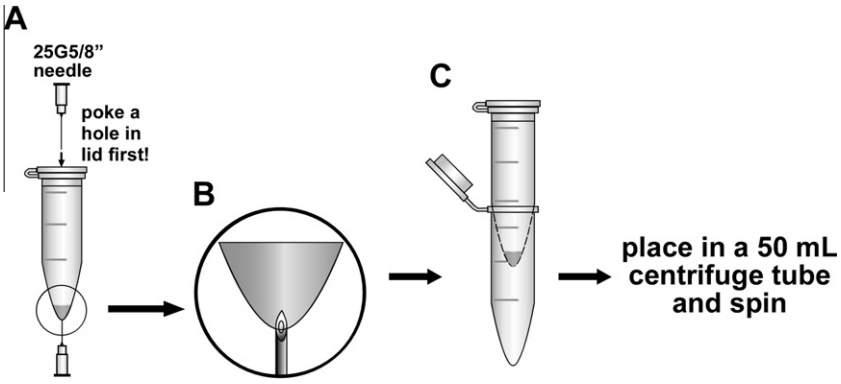

Fig. 2. A strategy for collecting eluted materials from the anti-Flag beads. (A) After cleaning the outside of an Eppendorf tube containing the anti-Flag beads and eluted materials (shown in gray at the bottom of the tube), a 25G5/8″ needle is used to FIRST poke a small hole in the lid of the tube and THEN a very small hole through the bottom of the tube. (B) An enlarged view showing that the beveled needle tip is inserted only halfway into the pointed end of the tube. (C) Insert the tube containing the beads into a new tube. Put the two-tube set into a 50 mL centrifuge tube and spin the liquid through the small hole into the bottom tube.

transcription assay, see below). The silver-staining and mass spectrometry procedures will be carried out essentially as described [26].

(B) Buffers

(1) *Buffer D0.3:* 20 mM Hepes, pH 7.9, 10% glycerol, 0.3 M KCl, 0.2 mM EDTA, 0.2% NP-40, 1 mM DTT*, and 0.5 mM PMSF* (*added fresh just before use).

(2) *Buffer D0.1:* 20 mM Hepes, pH 7.9, 10% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.2% NP-40, 1 mM DTT*, and 0.5 mM PMSF* (*added fresh just before use).

2.2. Functional analysis of the effects of P-TEFb-associated factors on HIV-1 transcription

The successful isolation and identification of novel factors in association with P-TEFb provide a unique opportunity for the subsequent functional characterization of their impact on basal and Tat-dependent HIV-1 transcription. Toward this goal, several experimental techniques such as the HIV-1 LTR-driven luciferase reporter assay and the chromatin immunoprecipitation (ChIP) assay have been used extensively in the field and described in great details in the literature. Despite their popularity, a major drawback of these assays is their inability to specifically examine the transcription process itself. For example, an increase in the HIV-1 LTR-driven luciferase activity can in theory be caused by an enhancement in the stability, nuclear export and/or translation of the luciferase mRNA. Likewise, the ChIP assay only detects the occupancy but not transcriptional activities of factors on a chromatin template. To overcome this deficiency, we describe here two techniques that will complement the well-known luciferase and ChIP methods and allow us to investigate the effects of P-TEFb and its associated factors on the production of HIV-1 transcripts in vitro and in vivo.

2.2.1. In vitro transcription assay

To investigate the effects of the affinity-purified P-TEFb and its associated factors on Tat-activated and TAR-dependent HIV-1 transcriptional elongation, an in vitro transcription assay employing two G-less transcription templates (HIV+TAR-G400 and HIVΔTAR-G100 plasmids, Fig. 3) present in a single reaction will be performed. These two templates were created by inserting the 400-bp (G400) and 100-bp (G100) G-less cassettes at a position approximately

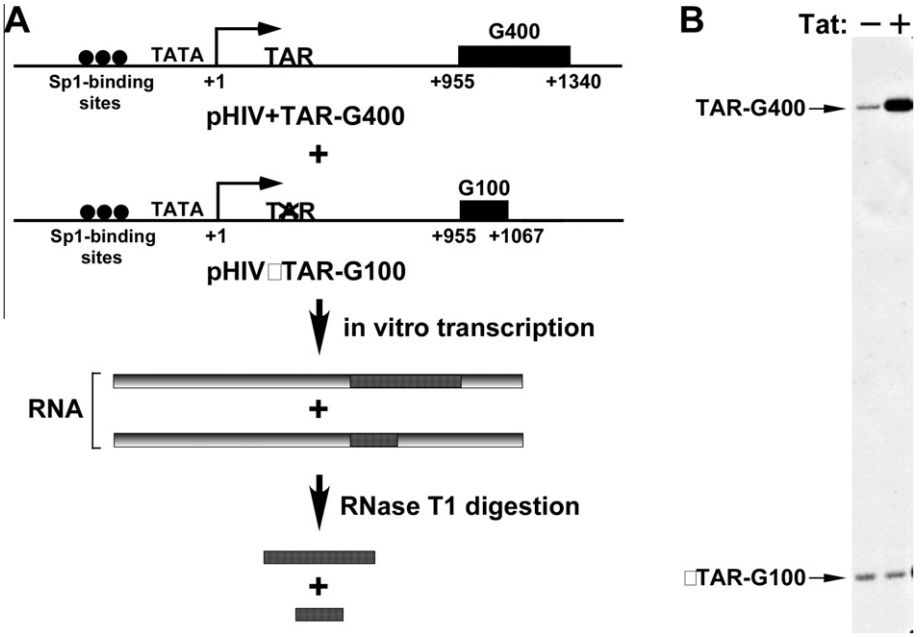

Fig. 3. An in vitro assay for measuring Tat-activated and TAR-dependent HIV-1 transcriptional elongation. (A) The ^{32}P-labeled RNA transcripts derived from two HIV-1 transcription templates, pHIV+TAR-G400 and pHIVΔTAR-G100, that are present in a single reaction are treated with RNase T1 to digest away all the RNA except for the 400-bp (G400) and 100-bp (G100) fragments transcribed from the two G-less DNA cassettes inserted at ~1-kb downstream of the HIV-1 transcription start site. The pHIVΔTAR-G100 template has a small deletion of the sequence encoding the TAR RNA element. (B) In vitro transcription reactions containing the two HIV-1 transcription templates, HeLa nuclear extract, and recombinant Tat protein (+) or buffer (−) were performed as in (A). The two ^{32}P-labeled G-less RNA fragments were isolated and analyzed by denaturing polyacrylamide gel and visualized by autoradiography.

1-kb downstream of the transcription start site within wild-type (+TAR) and the TAR-deleted (ΔTAR) HIV-1 LTR sequences, respectively (Fig. 3) [27]. Recombinant wild-type Tat protein and the C22G mutant that does not interact with P-TEFb will be purified as GST fusions from *Escherichia coli* and then cleaved off GST according to the described procedure [28]. The reactions also contain the mock- or CDK9-depleted HeLa NE in buffer D0.1 (prepared as described below). The affinity-purified P-TEFb together with its associated factors obtained in Section 2.1 will be added into the reactions to test their ability to complement the depleted NE and rescue HIV-1 transcription.

(A) Immunodepletion of CDK9 and its associated factors from HeLa NE

(1) The direct coupling method described by Harlow and Lane [29] is used to prepare the Protein A bead–anti-CDK9 antibody affinity resin (termed anti-CDK9 beads). This procedure allows the antibodies to be covalently cross-linked to Protein A via the bifunctional coupling reagent dimethylpimelimidate (DMP). We typically use 100 μg of the rabbit anti-CDK9 polyclonal antibodies (Santa Cruz Biotech, Cat. No.: sc-484) for every 100 μL of packed Protein A beads. For the purpose of setting up a mock depletion procedure later, a separate batch of fresh Protein A beads are processed the same way as the anti-CDK9 beads except that no antibodies are present in the coupling reaction.

(2) After cross-linking and extensive washing in buffer D0.5 containing 2 mg/mL BSA and 0.5% NP-40, the anti-CDK9 beads are divided equally into three Eppendorf tubes. Upon centrifugation for 15 s at 13,500 rpm, a Hamilton syringe fitted with a long needle is used to remove all traces of liquid from the packed beads. For mock depletion, the uncoupled Protein A beads were treated and divided similarly.

(3) Adjust the KCl and NP-40 concentrations of HeLa NE (~10 mg/mL) to 0.5 M and 0.5%, respectively, with concentrated solutions (e.g. 3 M KCl and 10% NP-40). Add 200 μL of the extract to the tube containing the anti-CDK9 beads and mix by gentle rotation for 45 min at 4 °C. Apply the same volume of NE to the uncoupled Protein A beads to start a mock depletion.

(4) At the end of the incubation, follow steps 6 and 7 described in Section 2.1 above to spin out all the liquid containing the depleted NE into the second tube containing fresh, unused anti-CDK9 beads (or uncoupled Protein A beads for mock depletion). Continue the depletion by gentle rotation for 45 min at 4 °C.

(5) Repeat step 4 to perform the third and last round of depletion. Once finished, spin out the mock- and CDK9-depleted NE, which are then dialyzed against buffer D0.1 and subjected to Western blotting with anti-CDK9 antibodies to determine the efficiency of depletion. Furthermore, Western analyses of irrelevant proteins such as α-tubulin and actin in the depleted NE can be conducted to confirm the specificity of CDK9 depletion. Finally, the depleted NE can also be analyzed by Western blotting with antibodies raised against the P-TEFb-associated factors to determine the extent of their co-depletion with CDK9. We have successfully used this approach to determine the fractions of nuclear HEXIM1 and LARP7 that are sequestered in the 7SK snRNP [11,12].

(B) Transcription reaction setup and conditions (*note:* the final reaction volume is 20 μL and contains ~60 mM KCl. Any variations of the final KCl concentration should be kept minimal and no more than 10%. Keep all ingredients on ice when setting up the reactions)

(1) Make a master mix so that each reaction will receive the following items in a total volume of 8 μL:

0.8 μL 125 mM $MgCl_2$
0.8 μL (αP^{32})-CTP (800 Ci/mmol)
0.15 μL RNasin (Promega; N2611)
0.75 μL poly(I)–poly(C) (0.35 mg/mL; GE, Cat. No.: 27-4732-01)
1.0 μL NTPs (3 mM ATP, 5 mM GTP, 5 mM UTP, 0.125 mM CTP)
0.5 μL DNA templates (50 ng/μL pHIV−TAR-G400 and 100 ng/μL pHIVΔTAR-G100)

0.2 μL DTT (300 mM)
3.8 μL H_2O (distilled and deionized; RNase-free).

(2) After mixing well all the ingredients, aliquot 8 μL from this master mix into a new tube. Then add to each tube 5.0 μL of either mock- or CDK9-depleted HeLa NE (~7 mg/mL) in buffer D0.1 (see above), 3.0 μL of the affinity-purified CDK9-F and associated factors prepared in buffer D0.1, 3.0 μL of buffer D0.1, and finally 1.0 μL of either recombinant Tat protein (25 ng/μL in D0.1) for the Tat(+) reactions or 1.0 μL of buffer D0.1 for the Tat(−) reactions [27].

(3) Briefly spin all the liquid to the bottom of the tube and mix by gentle tapping. Start the transcription reaction by incubating for 1 h at 30 °C.

(4) Add 3 μL RNase T1 (1 U/μL in buffer D0.1; Worthington Biochemical Corp., Cat. No.: LS01485) to each reaction, mix well and incubate for 5 min at 37 °C. RNase T1 cuts everything except for the G-less region in RNA transcripts transcribed from each of the two DNA templates. While the wild-type template contains a 400-bp G-less cassette, the ΔTAR template has a 100-bp G-less cassette. Therefore, after RNase T1 digestion, only two labeled RNA bands will appear on the gel (Fig. 3).

(5) Add 100 μL RNase stop solution containing proteinase K (Roche, Cat. No.: 03115879001) to each tube, mix and incubate for 10 min at 37 °C to digest away all the proteins and RNase T1.

(C) RNA isolation and analysis

(1) Add an equal volume of the mixture of phenol/chloroform (~120 μL) to each tube and vortex for about 20–30 s. Spin for 4 min at room temperature and transfer the top layer into a clean tube (make sure no phenol/chloroform is carried into the new tube).

(2) Add three volumes of ice-cold ethanol (~360 μL), invert the tubes several times and store at −20 °C for at least 15 min. Spin at 4 °C for 15 min and then carefully remove all the liquid without touching the very small whitish pellet attached to the very bottom of the tube.

(3) Wash the pellet once by slowly adding 0.5-mL 70% ethanol along one side of the tube and then spin again for 5 min at 4 °C. Remove the liquid as described above. This step removes most of the unincorporated (αP^{32})-CTP and thus reduces the background during autoradiography.

(4) Let the pellet dry by air or vacuum until it becomes completely invisible.

(5) Add 3 μL of the RNA loading buffer, vortex three times at 20 s each to dissolve the pellet and then spin briefly. Heat the samples for 1.5 min at 95 °C and then immediately put on ice.

(6) Run the samples through a 6% polyacrylamide/8 M urea sequencing gel. The dried gel can be analyzed by standard autoradiography with an X-ray film or a Phosphorimager.

(D) Buffers and solutions

(1) *Buffer D0.1:* 20 mM Hepes, pH 7.9, 10% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.2% NP-40, and 0.5 mM PMSF (add just before use).

(2) *Buffer D0.5:* 20 mM Hepes, pH 7.9, 10% glycerol, 0.5 M KCl, 0.2 mM EDTA, and 0.5 mM PMSF (added fresh just before use).

(3) *RNase stop solution:* 200 mM NaCl, 20 mM EDTA, 1% SDS, 40 μg/mL glycogen, and 80 μg/mL proteinase K (add just before use).

(4) *RNA loading buffer:* 90% formamide, 0.5× TBE electrophoresis buffer and 1× sequencing gel loading dye (Amresco, Cat. No.: E268-1mlAM).

2.2.2. In vivo transcription assay

To examine the effects of the P-TEFb-associated factors on HIV-1 transcription in vivo, a simple RT-PCR-based assay has been developed to examine the abundance of RNA transcripts at both the promoter-proximal and -distal regions (Fig. 4). Like Tat and P-TEFb, the P-TEFb-associated factors are expected to affect HIV-1 transcription at the stage of elongation through their control of P-TEFb and Tat activities. The ability of the in vivo assay to distinguish between promoter-proximal (i.e. transcriptional initiation) and -distal transcription (i.e. elongation) within the same cell and from a single HIV-1 LTR template provides an excellent opportunity to directly test the elongation activity of P-TEFb and its regulators. For this purpose, we routinely use an engineered HeLa cell line containing an integrated HIV-1 LTR-luciferase reporter gene [20], which has an added benefit of permitting a direct comparison between the affected luciferase RNA and protein levels within the same cell and under the same experimental conditions. The integrated HIV-1 LTR-driven luciferase reporter gene contains the 415-bp HIV-1 LTR sequence ending at +82 and the luciferase ORF starting at +104 relative to the HIV-1 transcription start site at +1 (Fig. 4).

(A) RNA isolation and treatment

(1) One day prior to transfection, 2×10^6 cells are freshly seeded in a 100 mm dish. The cells are transfected with either the cDNA constructs that overexpress the P-TEFb-associated factors or the shRNA-expressing plasmids that silence the expression of these factors. After another 36–48 h, total RNA are extracted from the transfected cells by using the Qiagen RNeasy mini kit (Cat. No.: 74106) according to the instructions provided by the vendor.

(2) Upon the elution of RNA from the spin column, transfer 50 μL of the RNA solution into a new 1.5 mL Eppendorf tube and add 10 μL of the DNase I digestion buffer, 10 μL of DNase I (1 U/μL; Fermentas, Cat. No.: EN0521), and 30 μL of RNase-free water. Upon incubation at 37 °C for 30 min, the reaction is stopped by adding 10 μL of 25 mM EDTA and incubating at 65 °C for 10 min. This is followed by standard phenol/chloroform extraction and ethanol precipitation. The RNA pellet is then dissolved in 50 μL RNase-free water and the RNA concentration is measured by UV spectrometer at 260 nm.

(B) RT-PCR

(1) In a 1.5 mL Eppendorf tube, add 2 μg RNA, 1 μL dNTP mix (10 mM each), 2 pmol gene-specific primer and RNase-free water

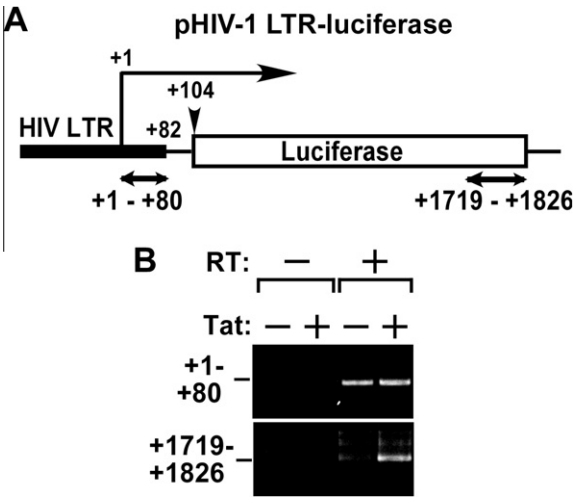

Fig. 4. An RT-PCR-based in vivo assay for distinguishing between promoter-proximal and -distal HIV-1 transcription. (A) The structure of the HIV-1 LTR-luciferase reporter gene, with +1 denoting the HIV-1 transcription start site. The thick line represents the 415-bp HIV-1 LTR sequence that ends at +82. The unfilled rectangle indicates the luciferase ORF that begins at +104. The two double-sided arrows represent the PCR-amplified DNA fragments corresponding to the promoter-proximal and -distal regions of the RNA transcripts. (B) mRNAs transcribed from the HIV-1 LTR-luciferase reporter gene and purified from cells expressing (+) or not expressing (−) Tat were subjected to RT-PCR analysis with primers that amplify the two indicated regions. RT, reverse transcriptase.

to a total of 12 μL. For the HIV-1 LTR-luciferase reporter gene, the reverse primer sequences used for first-strand cDNA synthesis of the promoter-proximal (HIV +1 to +80) and -distal (+1719 to +1826 relative to the HIV start site) regions are 5′-GTCACTTACCA AGCTTTATTGAG-3′ and 5′-TTTGGACTTTCCGCCCTT-3′, respectively.

(2) Heat the mixture to 65 °C for 5 min and chill immediately on ice. Collect the contents of the tube by brief centrifugation and add 4-μL 5× first-strand buffer, 2-μL 0.1 M DTT and 1 μL RNasin. Mix contents of the tube gently and incubate at 42 °C for 2 min. Add 1 μL (200 U) of SuperScript™ II reverse transcriptase (RT, Invitrogen, Cat. No.: 18064-014), mix gently, and incubate at 42 °C for 50 min to produce the first-strand cDNA. Inactivate the reaction by heating at 70 °C for 15 min.

(3) Pipet out 5 and 10 μL cDNA as template for PCR amplification of the promoter-proximal and -distal regions, respectively. Add 4-μL 10× PCR buffer, 1.6 μL dNTP mix (5 mM each), 1.6 μL PCR primer set (5 μM each), Taq DNA polymerase (5 U/μL), and distilled water to the final volume of 40 μL. The forward primer sequences used for PCR amplification of the HIV-1 promoter-proximal (HIV +1 to +80) and -distal (+1719 to +1826 relative to the HIV start site) regions are 5′-CGAGTTAGACCAGATCTGAGCC-3′ and 5′-GGAGGAGTTGTGTTTGTGGAC-3′, respectively.

4. Heat the reactions to 94 °C for 3 min to denature, perform 23–25 cycles of PCR (94 °C, 30 s; 52 °C, 30 s; 72 °C, 20 s), and then follow by extension at 72 °C for 10 min. Load 10 μL of the amplified samples with DNA dye into a 2% agarose gel. A real experiment employing this RT-PCR-based approach to demonstrate Tat activation of the HIV-1 promoter-distal but not -proximal transcription is shown in Fig. 4.

(C) Buffers

(1) *DNase I digestion buffer:* 100 mM Tris–HCl (pH 7.5), 25 mM $MgCl_2$, and 1 mM $CaCl_2$.

(2) *5× first-strand buffer:* 250 mM Tris–HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$.

(3) *10× PCR buffer:* 200 mM Tris–HCl (pH 8.8), 100 mM $(NH_4)_2SO_4$, 100 mM KCl, 20 mM MgSO4, 1% Triton X-100.

3. Concluding remarks

Ever since the identification of P-TEFb more than a decade ago as a specific host cellular co-factor for Tat activation of HIV-1 transcription [1–3], this landmark discovery has provided the basic framework for our understanding of Tat function in the HIV-1 life cycle. However, almost immediately after the discovery of this important link between P-TEFb and Tat-transactivation, evidence has begun to emerge to show that P-TEFb never acts alone in the cell. Subsequent studies from many laboratories including our own have vastly expanded our knowledge about how P-TEFb itself is regulated in vivo by a rather large number of associated factors and how these P-TEFb regulators can impact on basal and Tat-activated HIV-1 transcription. The set of experimental approaches described here have contributed significantly to our efforts to isolate and identify several P-TEFb-associated factors and investigate their roles in Tat/P-TEFb-mediated activation of HIV-1 transcription. As it is highly likely there exist additional yet to be identified P-TEFb-associated factors that can further modulate HIV-1 transcription in conjunction with P-TEFb and Tat, the methods described here should prove to be very useful for our future identification and characterization of these new factors. It should be pointed out that although the various methods described here were initially developed to study P-TEFb and its associated factors and their control of HIV-1 transcription, they can be adapted easily for future investigations of other unrelated protein complexes and/or control of transcriptional elongation of other unrelated genes. This latter application can be especially useful and timely given the recent demonstrations that promoter-proximal pausing of RNA Pol II and its subsequent elongation play a much more important role in controlling the expression of a very large number of metazoan genes than previously thought [30,31].

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