C-terminal Domain Phosphatase Sensitivity of RNA Polymerase II in Early Elongation Complexes on the HIV-1 and Adenovirus 2 Major Late Templates

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The fate of RNA polymerase II in early elongation complexes is under the control of factors that regulate and respond to the phosphorylation state of the C-terminal domain (CTD). Phosphorylation of the CTD protects early elongation complexes from negative transcription elongation factors such as NELF, DSIF, and factor 2. To understand the relationship between transcript elongation and the sensitivity of RNA polymerase IIO to dephosphorylation, elongation complexes at defined positions on the Ad2-ML and human immunodeficiency virus type 1 (HIV-1) templates were purified, and their sensitivity to CTD phosphatase was determined. Purified elongation complexes treated with 1% Sarkosyl and paused at U14/G16 on an HIV-1 template and at G11 on the Ad2-ML template are equally sensitive to dephosphorylation by CTD phosphatase. Multiple elongation complexes paused at more promoter distal sites are more resistant to dephosphorylation than are U14/G16 and G11 complexes. The HIV-1 long terminal repeat and adenovirus 2 major late promoter do not appear to differentially influence the CTD phosphatase sensitivity of stringently washed complexes. Subsequent elongation by 1% Sarkosyl-washed U14/G16 complexes is unaffected by prior CTD phosphatase treatment. This result is consistent with the hypothesis that CTD phosphatase requires the presence of specific elongation factors to propagate a negative effect on transcript elongation. The action of CTD phosphatase on elongation complexes is inhibited by HIV-1 Tat protein. This observation is consistent with the idea that Tat suppression of CTD phosphatase plays a role in transactivation.

The regulation of transcript elongation plays an important role in the control of gene expression. Elongation in eukaryotes is catalyzed by RNA polymerase IIO and is regulated in multiple ways. One mechanism involves the transition of early elongation complexes (EECs) into a processive or productive elongation mode. This choice is influenced by positive and negative transcription elongation factors designated P-TEFb and N-TEF, respectively. This effect is mediated by the phosphorylation state of the C-terminal domain (CTD) of the largest RNA polymerase II subunit, P-TEFb, and negative factors such as DSIF (DRB sensitivity-inducing factor), NELF (negative elongation factor), and factor 2 (1–6). A second way of controlling elongation is by factors that influence the overall elongation rate of RNA polymerase IIO. Factors involved in this level of control include TFIIF, SII, elongin, and the ELL proteins (reviewed in Ref. 7).

The factors that control the entry into productive elongation, P-TEFb, NELF, and DSIF, affect or are sensitive to the phosphorylation state of the CTD. The CTD, which is composed of repeats of the consensus sequence YSPTSPS, occurs in two major forms. The unphosphorylated form of RNA polymerase IIA, assembles into the preinitiation complexes, while the highly phosphorylated form, IIO, is associated with active elongation complexes (reviewed in Ref. 8). Protein kinases and phosphatases that act upon the CTD can thus be stimulatory or inhibitory, depending upon where they act in the transcription cycle. A CTD kinase that phosphorylates the CTD of free polymerase decreases the pool of RNA polymerase IIA available for initiation and hence could function as a global negative regulator of transcription. The SRB 10/11 kinase appears to function in this manner (9). Conversely, a CTD phosphatase that dephosphorylates free RNA polymerase IIO, thereby increasing the pool of RNA polymerase IIA, should have the opposite effect. A CTD phosphatase has been described that actively dephosphorylates free RNA polymerase IIO (10–13). CTD kinase(s) and phosphatase(s) acting on RNA polymerase IIO in an elongation complex would be expected to have opposite effects. P-TEFb was initially described based upon its activity as an elongation factor in nuclear extracts (1, 14). It was later found that P-TEFb is a CTD kinase that acts on EECs to promote their entry into productive elongation (15). Conversely, CTD phosphatase that acts on EECs in the presence of NELF and DSIF would result in an inhibition of transcript elongation (5, 16).

One of the most prominent and well studied examples of regulation at the level of transcript elongation is the expression of the HIV-1 genome. In the absence of the HIV-1 Tat protein, initiation at the LTR gives rise to elongation complexes with limited processivity. In the presence of Tat, EECs are converted to a highly processive form. Tat mediates transactivation through an element in the nascent RNA called the transactivation response (TAR) element (reviewed in Refs. 17 and 18). Both P-TEFb and an intact CTD are required for efficient transactivation.
transactivation (19–26). P-TEFb is a heterodimer of the cyclin-dependent kinase Cdk9 and cyclin T (27). Cyclin T exists in at least three forms, T1, T2a, and T2b. T1 is the most prevalent form and is probably involved in Tat transactivation (28). Tat interacts strongly with T1, and this complex binds efficiently to the TAR RNA (25). Presumably Tat recruits P-TEFb to the TAR element, where it can phosphorylate the CTD of the transcribing polymerase, thus leading to activation. Since P-TEFb is a general factor and even supports transcription from the LTR in the absence of Tat (29, 30), it is not clear why it must be so strongly recruited to the LTR for transactivation. Perhaps TFIIH does not efficiently phosphorylate the CTD at the LTR. Although early evidence suggests that TFIIH plays a role in transactivation (19, 31), more recent studies indicate that CAK is dispensable for transactivation (32). Alternatively, the EECs originating from the HIV-1 LTR may be more susceptible to dephosphorylation, thereby generating a requirement for the efficient recruitment of P-TEFb (16).

There is only one known protein phosphatase capable of selective dephosphorylation of the CTD. The gene for CTD phosphatase has been cloned in both humans and yeast and has been termed FCP1 (10, 13, 33, 34). In humans, CTD phosphatase activity is contained within the 150-kDa polypeptide, with no other protein required for activity (16). However, CTD phosphatase is strongly stimulated by the basal transcription factor TFIIH. The RAP74 subunit of TFIIH is fully competent to stimulate CTD phosphatase activity in vitro (12). The stimulatory action of RAP74 is inhibited by the basal transcription factor TFIIB. CTD phosphatase can dephosphorylate RNAP IIo elongating on c5-terminal templates with an efficiency comparable with that of free RNAP IIo (35). In addition, CTD phosphatase can dephosphorylate EECs generated in partially or highly purified, RNAP II-dependent transcription systems (34, 35). Tat interacts directly with CTD phosphatase (33) and inhibits both the basal and RAP74-stimulated activity of CTD phosphatase (16). This has led to the hypothesis that Tat can interfere with CTD phosphatase action on EECs, thus providing a protective effect.

These studies demonstrate that EECs located proximal to the promoter on both Ad2-ML and HIV-1 LTR templates are sensitive to CTD phosphatase, whereas complexes that are more distal are relatively resistant to dephosphorylation. This change takes place at approximately nucleotide +25 on both templates. Besides inhibiting the dephosphorylation of free RNAP IIo, Tat inhibits the ability of CTD phosphatase to dephosphorylate RNAP IIo in EECs initiated from the HIV-1 LTR. Finally, the state of CTD phosphorylation does not appear to influence the elongation efficiency of complexes treated with 1% Sarkosyl. These results are consistent with the idea that CTD phosphatase requires the activity of extrinsic elongation factors to exert a negative influence on elongation.

**EXPERIMENTAL PROCEDURES**

**Materials and Buffers**—The Mono Q column and radiolabeled [α-32P]CTP (800 Ci/mmol) were purchased from Amersham Pharmacia Biotech. Streptavidin-coated Dynabeads M-280 were from Dynal Inc. Transcription template TAR-G400 was provided by Philip Sharp (Massachusetts Institute of Technology). Taq DNA polymerase (5 units/μl) was from Roche Molecular Biochemicals. TE buffer contains 50 mM Tris-HCl, pH 7.9, 0.1 mM EDTA. HMKS contains 20 mM HEPES, 7 mM MgCl2, 55 mM KCl, 0.1% Triton X-100. HMKS is the same composition as HMKT except that it contains 1% Sarkosyl in place of Triton X-100. CTD phosphatase buffer contains 50 mM Tris, pH 7.9, 10 mM MgCl2, 20% glycerol, 0.025% Tween 80, 0.1 mM EDTA, and 5 mM dithiothreitol added just before use.

**PCR Template Preparation**—Template DNA containing the HIV-1 LTR, termed 5’-BIO-PCR1 was synthesized using standard PCR technologies. The sequence of the upstream primer was 5’-TCTTCCAGGGTTTGAAGTTCC-3’, and the sequence of the downstream primer was 5’-TCTTCCAGGGTTTGAAGTTCC-3’. The upstream primer was biotinylated at its 5'-end through an MBS linkage. For a typical preparation of 5’-BIO-PCR1, 2 μl of PCR (in 20 100-μl aliquots) was assembled in 1× Roche Molecular Biochemicals PCR buffer (1.5 mM MgCl2) with 0.2 μM concentration of each NTP. This 2-μl reaction contained both primers at 1 μM and 2 μl of 500-nucleotide TAR-G400 DNA (0.5 pmol). This amplifies an 873-base pair DNA template that contains the wild-type HIV-1 LTR promoter and TAR sequence and produces a run-off transcript of 533 nucleotides. After PCR, the 2 ml of amplified DNA mixture was phenol-extracted and ethanol-precipitated. Pellets were resuspended in 250 μl of TE and loaded onto a 1-ml Mono Q column. The Mono Q column was eluted with a 20-ml linear gradient of 0.1–1.0 M NaCl in TE. The peak of 5’-BIO-PCR1 eluted at approximately 0.75 M NaCl. The Ad2-ML template was prepared as described previously (35). Peak fractions were bound directly to Dynabeads M-280 at 3.5 μg of DNA/mg of beads for 55 min at 24°C. Beads with immobilized template were washed twice in TE and then stored at 4°C. The final DNA concentration was ~40 μg/ml of bead slurry.

**Transcription on Immobilized Templates**—To form preinitiation complexes on immobilized templates, 5 μl of HeLa nuclear extract (~73 μg of protein) was incubated for 30 min at 30°C in a 12-μl reaction containing 20 mM HEPES, pH 7.9, 7 mM MgCl2, 55–60 mM KCl, 7 mM dithiothreitol, and 100 ng of immobilized template on beads. HeLa nuclear extract was prepared as described previously (37). For transcription of 5’-BIO-PCR1, the pulse labeling of transcripts was initiated by the addition of 2 μl of nucleotide solution, which brought the concentration to 600 μM dATP, 200 μM GTP, 200 μM UTP, and 0.6 μM [α-32P]CTP (~5 μCi). For transcription of the Ad2-ML template, the nucleotide concentration for pulse conditions was 600 μM ATP, GTP, and UTP and 0.6 μM [α-32P]CTP. For both templates, labeling was for 30 s at 30°C and was terminated by the addition of 0.5 μl of 0.5 M EDTA. Beads containing the stalled EECs were then magnetically concentrated and washed once with 12 μl of HMKT. The beads were successively washed twice with 12 μl of HMKS and then twice with 12 μl of HMKT. EECs were then resuspended in 12 μl of HMKT. Elongation complexes were walked down the template by the addition of 2 μl of solution containing the required nucleotides to give a final concentration of 20 μM for each nucleotide present. After 5 min at 30°C, the complexes were magnetically concentrated, washed twice with 12 μl of HMKT, and resuspended in 12 μl of HMKT. This cycle of chase-wash-resuspension was repeated as many times as needed to advance complexes to the intended position. For reactions containing 32P-labeled RNA IIa, polymerase was incubated on ice for 10 min with the HeLa extract before the addition of template DNA. 32P-labeled RNA IIa was prepared as described previously (16). In reactions containing 32P-labeled RNA IIa, [α-32P]CTP was replaced with cold CTP.

**Purification of CTD Phosphatase**—CTD phosphatase was purified as described previously (16). Purified CTD phosphatase had a specific activity of 9000 units/mg and a concentration of ~4 units/μl. One unit of CTD phosphatase corresponds to the activity required to dephosphorylate 1 pmol of free RNAP IIo in 1 min in the presence of a saturating amount of RAP74 (11).

**CTD Phosphatase Assays**—CTD phosphatase assays with free RNA IIo as substrate were performed as described previously except that the dithiothreitol concentration was 5 mM (11). 32P-labeled RNA IIo was prepared as described previously (16). For assays involving isolated EECs, 24 μl of complex (twice the standard transcription reaction) in HMKT were washed twice with 20-μl aliquots of CTD phosphatase buffer and resuspended in 8 μl of phosphatase buffer. Aliquots (2 μl) of CTD phosphatase buffer containing RAP74 and the indicated amounts of CTD phosphatase were then added. For chase reactions, complexes were washed twice with 24 μl of HMKT and resuspended in 24 μl of HMKT. Transcription was resumed by adding 4 μl of a solution containing all four NTPs to each 24 μl of complexes. Reaction volumes for the analysis of labeled RNA II were twice the volume of reactions to monitor transcript length. Assays were quantitated by exposing gels to a Fuji imaging plate that was then read on a Fuji phosphor imager or a Storm imager. Approximately equal sized gel areas were defined for background subtraction and IIA and IIIo bands as well as intermediate forms. Lane-specific backgrounds were taken from just above the IIIo band. Typically, a reference standard equivalent to 2.5% of the input 32P-labeled RNA IIa was taken from the preincubation mix just before the addition of nucleotides.

**RESULTS**

Synchronized EECs Can Be Formed on Immobilized Templates in HeLa Nuclear Extracts—To determine if CTD phosph...
phatase can act on EECs that are formed under conditions that allow Tat transactivation, it was first necessary to adapt the immobilized template transcription protocol for use in a HeLa nuclear extract. An immobilized template based on an HIV-1 DNA (36) was constructed (see "Experimental Procedures"). Preinitiation complexes were formed on the HIV-1 (5'-BIO-PCR1) template and subjected to brief labeling in the presence of a limiting set of nucleotides. In all cases when ATP was included, the transcripts generated were nonsynchronous even after a short pulse (data not shown). To avoid this problem, dATP was used to provide the energy requirement for initiation. The condition that gave the best combination of efficiency and synchronization was a 30-s pulse including dATP, GTP, UTP, and limiting CTP followed by stalling with EDTA. The EECs were then rapidly isolated with a magnet and washed with buffer containing 1% Sarkosyl followed by buffer in the absence of detergent. The result was a mixture of complexes stalled at positions U14 and G16 (Fig. 1A, lane 1). As can be seen from the sequence information at the top of Fig. 1A, transcripts that extend to U14 have already passed the site for the incorporation of an A residue at position +11, and complexes at G16 have passed the site of incorporation of A15 as well. Whether this observation is the result of contamination of one or more of our NTP solutions with ATP, misincorporation of another nucleotide, or the incorporation of dAMP residues into the transcript is unknown. The U14/G16 complexes can be "walked" to various discrete points by alternating incubations with sets of three NTPs. The stringently washed EECs can elongate with reasonable efficiency out to position U46. However, after reaching U46, subsequent walking of complexes is not efficient (Fig. 1A, lanes 7–12). Therefore, up to nucleotide 46, discrete and relatively homogenous populations of EECs can be isolated on the HIV-1 template.

In order to have appropriate controls for gene-specific effects, the immobilized Ad2-ML template described previously (35) was tested in the HeLa nuclear extract system. Surprisingly, the synchronization of the EECs on this template was better than that observed for the HIV-1 template. As shown in Fig. 1B (lane 1), a 30-s pulse in the presence of ATP, GTP, UTP, and limiting CTP results in the synchronized progression of elongation complexes to position 11. These stringently washed EECs can also be walked to discrete sites on the Ad2-ML template with limited chase reactions (Fig. 1B, lanes 2–12). Importantly, the transcription of promoter-proximal sequences of the Ad2-ML template does not result in multiple pause sites as observed on the HIV-1 template. The first site that causes a significant fraction of polymerases to pause is between nucleotides A71 and U91 (Fig. 1B, lanes 9–12). These results indicate that EECs stalled at nearly any point in the early transcribed region of Ad2-ML can be prepared with a high level of purity.

HeLa Nuclear Extract Can Incorporate Exogenous Labeled RNAP II into EECs—Previous experiments to examine the activity of CTD phosphatase on elongating RNAPII relied on RNAPII-dependent reconstituted transcription systems (34, 35). These systems are incapable of supporting Tat transactivation and thus are not good model systems for the analysis of transcript elongation. To monitor the level of CTD phosphorylation in the unfraccionated system capable of Tat transactivation, the nuclear extract was supplemented with 32P-labeled RNAPII and incubated briefly before the addition of template. Under these conditions, in the absence of [α-32P]CTP, labeled RNAPII was incorporated into EECs. Treatment of EECs with 1% Sarkosyl results in complexes that contain an equimolar amount of RNAPII and transcript (35). This stringent wash ensures that all of the labeled RNAPII recovered is in active transcription complexes. The RNAPII assembled into preinitiation complexes was almost completely converted to RNAPIIO by the pulse reaction on both the HIV-1 and the Ad2-ML templates (Fig. 2, compare lanes 7 and 13 with lanes 8 and 14, respectively). While the template-bound RNAPII was efficiently converted from IIA to IIO, the free RNAPII remained as IIA (Fig. 2A, lanes 7 and 13). The average recovery of labeled RNAPIIO as a function of input RNAPII varies between 0.5 and 2.0% for both templates. In Fig. 2A, the average recovery is 1.7% for HIV-1 EECs (lanes 8–12) and 1.3% for Ad2-ML EECs (lanes 14–18).

Stringently Washed EECs Are Sensitive to CTD Phosphatase—To determine if CTD phosphatase can act on HIV-1 EECs, complexes paused at positions U14/G16 were washed with buffer containing 1% Sarkosyl followed by CTD phosphatase buffer and incubated with RAP74 alone or RAP74 plus increasing amounts of CTD phosphatase (Fig. 2A, lanes 9–12). Parallel CTD phosphatase reactions on complexes paused on the Ad2-ML template at position G11 were also performed.
CTD Phosphatase Sensitivity of RNA Polymerase II

(Fig. 2A, lanes 15–18). RNAP IIO contained in complexes on both templates is sensitive to dephosphorylation by CTD phosphatase. However, elongation complexes on both HIV-1 and Ad2-MLP templates are significantly less sensitive to dephosphorylation than is free RNAP IIO (Fig. 2A, compare lanes 3–6 with lanes 9–12 and lanes 15–18). Quantitation of these results indicates that the sensitivity of complexes on the HIV-1 and Ad2-MLP templates is comparable (Fig. 2B). A convenient measure of complex sensitivity can be derived from the quantitation of subunits IIo and IIa as a function of CTD phosphatase concentration. The point at which the percentage of subunits IIa and IIo are equal, which is read as the cross-over point (or 50% conversion point) in Fig. 2B, is a reliable measure of sensitivity. This crossover point is at less than 4 milliunits for free RNAP II. The crossover point is about 400 milliunits for the pulse ECCs on both the HIV-1 and Ad2-ML templates (center and right graphs of Fig. 2B). This indicates that the template-bound RNAP IIO in those complexes is about 2 orders of magnitude less sensitive to dephosphorylation than is free RNAP IIO. The increased resistance of RNAP IIO contained in ECCs does not result from an inhibition of CTD phosphatase activity by DNA or paramagnetic beads (35).

Promoter-proximal Complexes Are More Sensitive to Dephosphorylation than Are Distal Complexes—To understand the role CTD phosphatase might play in early elongation, it is necessary to map the sensitivity of complexes as a function of their position on the template. HIV-1 ECCs paused at nucleotides G26, G36, and U46 were isolated, and their sensitivity to dephosphorylation was determined by incubation with increasing amounts of CTD phosphatase. The results were quantified as described above and graphed as a function of milliunits of CTD phosphatase (Fig. 3A). The analysis of each complex included U11/G16 as an internal control. Elongation complexes at positions G26, G36, and U46 are all less sensitive to dephosphorylation than are complexes at position U3/G16. As control for template specific effects, complexes at comparable positions, G25, C34, and A48, were isolated from transcription of the early region of the Ad2-ML template (Fig. 3B). As on HIV-1, all Ad2-ML complexes at and downstream from G25 are less sensitive to CTD phosphatase than are complexes at G31. A representative panel of the gel analysis, which includes HIV-1 complexes at position U3/G16 and Ad2-ML complexes at A48, is shown in Fig. 3C.

Tat Inhibits the Activity of CTD Phosphatase on ECCs—HIV-1 Tat protein inhibits the activity of CTD phosphatase when free RNAP IIO is the substrate (16). To determine if Tat is capable of inhibiting the action of CTD phosphatase on ECCs, U11/G16 ECCs were treated with CTD phosphatase in the presence of increasing concentrations of Tat (Fig. 4). As reported previously (16), Tat is an effective inhibitor of CTD phosphatase (Fig. 4, lanes 3–8). This effect was seen despite the large excess of CTD phosphatase over what is minimally required to dephosphorylate the RNAP IIO present (compare Fig. 4, lane 3, with Fig. 3C, lanes 3–6). In parallel reactions, 100

![Image](https://example.com/image.png)
Fig. 3. CTD phosphatase sensitivity of promoter-proximal and -distal EECs. A, a quantitation of CTD phosphatase activity on elongation complexes paused at positions U14/G16, G25, G34, and A48 on the HIV-1 template. B, a similar analysis of complexes paused at positions G11, G25, C34, and A48 on the Ad2-ML template. The results presented in the far left graphs of A and B are the average of three experiments. C is a representative 5% SDS-PAGE analysis of CTD phosphatase reactions with free RNAP IIO or EECs as substrate. The left panel of C shows the products of the CTD phosphatase reaction for free RNAP IIO (lanes 3–6), U14/G16 complexes (lanes 8–11), and U46 complexes (lanes 13–16) on the HIV-1 template. Lanes 1 contain 2.5% of the input 32P-labeled RNAP IIA used to generate the EECs in other lanes. Lanes 2, 7, and 12 contain samples of RNAP IIO, U14/G16, and U46 complexes prior to addition of CTD phosphatase/RAP74, respectively. The left panel of C shows the comparable analysis of complexes on the Ad2-ML template. U14/G16, G25, G34, and U46 HIV-1 EECs were generated as in Fig. 1A, with the addition of 32P-labeled RNAP IIA. G11, G25, C34, and A48 Ad2-ML EECs were generated as in Fig. 1B. Purified complexes were incubated with varying amounts of purified CTD phosphatase. Elongation complexes on the HIV-1 template contained 2.3–2.8, 1.6, 2.3, and 2.1 fmol of 32P-labeled RNAP IIA. G11, G25, C34, and A48 Ad2-ML EECs were generated as in Fig. 1C. Purified complexes were incubated with varying amounts of purified CTD phosphatase. Elongation complexes on the Ad2-ML template contained 0.67–0.87, 0.74, and 0.60, and 0.56 fmol of 32P-labeled RNAP IIO for complexes positioned at G11, G25, C34, and A48, respectively. The positions of subunits IIA and I0 are as indicated. mU, milliunits.

DISCUSSION

These studies establish that the sensitivity of paused elongation complexes to dephosphorylation is influenced by their
position relative to the transcriptional start site. Although the nature of the template does not appear to influence the sensitivity of paused elongation complexes, the frequency and duration of pausing are template-specific. Utilizing an immobilized template, EECs can be moved to almost any desired position on the early transcribed region of the HIV-1 template through a combination of the appropriate restricted nucleotide chase regimens. A major obstacle in the transcription of the first 90 nucleotides of the HIV-1 template is the strong pause at approximately nucleotide U146. High levels of pausing and premature termination result during transcription from the HIV-1 LTR in the absence of Tat (17, 38). Although this behavior is linked to the TAR RNA sequence, the site of pausing or termination is condition-dependent (39–42). It is possible that the TAR RNA sequence, the site of pausing or termination result during transcription from the HIV-1 template, is probably responsible for determining where abortive transcripts terminate. Interestingly, only about 40% of the elongation complexes make it past the major pause site at U146 on the HIV-1 template. In contrast, a comparable drop off in elongation efficiency on the Ad2-ML template occurs only after nucleotide 92. Other factors being equal, this result suggests that it is more difficult for EECs to transit the promoter-proximal region on the HIV-1 template than the Ad2-ML template.

Previous experiments have established that CTD phosphatase can dephosphorylate RNAP IIO contained in elongation complexes (34, 35). Although these studies suggest a link between the distance of an elongation complex from the promoter and the sensitivity of RNAP II to dephosphorylation (35), results presented here are the first fine-scale dissection of EEC sensitivity. Promoter-proximal complexes, within 16–25 nucleotides from the transcriptional start site, are relatively sensitive to CTD phosphatase although substantially more resistant than free RNAP IIO (Fig. 2). On both the HIV-1 and Ad2-ML templates, these promoter-proximal complexes are appreciably more sensitive to dephosphorylation than are complexes that have moved past nucleotide +25. Elongation from position +25 to positions 46–48 does not result in an appreciable change in CTD phosphatase sensitivity (Fig. 3). This is reminiscent of the change in the protein-DNA footprint of elongation complexes as a function of their position on the template (46). In that study, complexes undergo a dramatic shift in the size and location of their footprints relative to the length of the nascent RNA between +20 and +30 on a version of the Ad2-ML promoter. As EECs transit this region, the footprint lags behind the site of nucleotide addition, remaining more promoter-proximal. At some point, the footprint appears to “snap” forward to become more centered around the nucleotide addition site. The conformational change observed in footprint analysis may be related to changes in phosphatase sensitivity. Whatever the mechanism, it presumably is a rearrangement or isomerization of the RNAP II itself and is not likely to be dependent on associated factors. Treatment of elongation complexes with 1% Sarkosyl would most likely dissociate such factors.

An objective in initiating this series of experiments was to test the hypothesis that premature termination of transcripts initiated in the HIV-1 LTR was the consequence of the dephosphorylation of RNAP IIO (16). Under the conditions of these experiments, the sensitivity of stringently washed paused complexes on the HIV-1 template is not enhanced relative to complexes paused at comparable positions on the Ad2-ML template. Accordingly, there is not a simple correlation between the frequency of abortive transcription, which is high in the case of transcription from the HIV-1 promoter and low for transcription from the Ad2-ML promoter, and the sensitivity of RNAP IIO in paused complexes to dephosphorylation. Although there is no apparent difference in sensitivity within the region examined, the possibility that HIV-1 EECs pass through a region downstream of U146, where their sensitivity is inherently higher can not be eliminated. Another possibility is that treatment of elongation complexes with 1% Sarkosyl removes a factor(s) that can mediate the sensitivity of elongation complexes (35). Finally, the observation that there are multiple pause sites in early regions of the HIV-1 template, relative to the Ad2-ML template, suggests it may take a significantly longer time for RNAP II to transcribe promoter-proximal sequences in the HIV-1 template. Accordingly, although the paused complexes on both templates have comparable sensitivities, more dephosphorylation of RNAP IIO will occur on the HIV-1 template simply because complexes spend more time traversing the region where RNAP II is most sensitive.
The observation that Tat inhibits the activity of CTD phosphatase on HIV-1 EECs (Fig. 4) is consistent with the idea that CTD phosphatase plays a direct role in transactivation (16). However, in the case of U14/G16 EECs, not enough of the Tat binding site present in TAR-containing RNA is exposed from the RNAP II to allow binding. It is unknown whether the Tat inhibition is taking place by the binding and inactivation of free CTD phosphatase or if some type of ternary complex is formed on RNAP II. CTD phosphatase is known to interact directly with a site on RNAP II that is distinct from the CTD (12). Furthermore, Tat interacts directly with CTD phosphatase (16, 33). Further studies are necessary to define the mechanism of Tat inhibition.

It has recently been shown that P-TEFb, artificially recruited to an RNA target, is sufficient to generate a high level of transcription, even in the absence of Tat (47). Since this method results in a very high local concentration of P-TEFb, it is likely that any negative regulators like CTD phosphatase, which might play roles under more normal cellular conditions, would escape detection. Clearly the level of CTD phosphorylation is determined by the relative activity of CTD kinase(s) and CTD phosphatase. A major increase in the localized concentra-
tion of either enzyme would be expected to have a profound effect on transcription. The natural occurrence of the TAR sequence places the Tat/P-TEFb complex in the correct orientation relative to the CTD to allow proper function. It is also possible that Tat may be in a position to influence the activity of other CTD-interacting factors.

The elongation properties of RNAPII on the HIV-1 template are unaffected by the state of phosphorylation of the CTD (Fig. 5). The RNAPII in EECs was not capable of rephosphorylation under the assay conditions presumably because CTD kinases were removed by the wash conditions. Although CTD phosphatase has been reported to act as a weak positive elongation factor (34), no positive effect on elongation was seen in these studies. However, since EECs treated with CTD phosphatase were washed thoroughly before elongation was allowed to resume, the amount of CTD phosphatase remaining might be insufficient to promote elongation. Stringently washed complexes are also missing termination factors such as factor 2 (2, 3) and negative elongation factors like NELF (5) and DSIF (4, 6). Accordingly, it is not surprising that they elongate reasonably well. However, since the positive elongation factors such as SII and TFIIF are also missing, strong pause sites are more likely to be a barrier than a promoter. These results suggest that, like dC-tailed template EECs (5), stringently washed EECs elongate independently of the state of CTD phosphorylation.

The observation that the CTD phosphatase sensitivity of RNAPII in early elongation complexes depends on its position within the transcription unit may have important regulatory implications. The time required for RNAPII to move through regions of greatest sensitivity is template-specific and could in principle influence the extent of RNAPII dephosphorylation in a gene-specific manner. Future studies will focus on testing this hypothesis and establishing the role of extrinsic factors in determining the sensitivity of RNAPII to CTD phosphatase. It is also important to examine the sensitivity of RNAPII in a dynamic system, since the conformation of a paused polymerase is probably different from that of an actively elongating enzyme.

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