Trans-activation by human immunodeficiency virus Tat protein requires the C-terminal domain of RNA polymerase II

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ABSTRACT Human immunodeficiency virus (HIV)-encoded trans-activator (Tat) acts through the transcriptional response element RNA stem-loop to increase greatly the processivity of RNA polymerase II. Without Tat, transcription originating from the HIV promoter is attenuated. In this study, we demonstrate that transcriptional activation by Tat in vivo and in vitro requires the C-terminal domain (CTD) of RNA polymerase II. In contrast, the CTD is not required for basal transcription and for the formation of short, attenuated transcripts. Thus, trans-activation by Tat resembles enhancer-dependent activation of transcription. These results suggest that effects of Tat on the processivity of RNA polymerase II require proteins that are associated with the CTD and may result in the phosphorylation of the CTD.

Trans-activator (Tat) of primate and equine lentiviruses is a unique RNA-binding transcriptional activator (1). Through its interactions with trans-activation response element (TAR), a hairpin structure that forms in the 5'-untranslated region of all viral RNAs, Tat increases rates of elongation by RNA polymerase II (2, 3). Although its mechanism of action is still unknown, several studies have suggested direct roles for RNA polymerase II, general transcription factors, and various transcriptional co-activators in Tat trans-activation (4–7). At present, it is not known whether effects of Tat resemble enhancer-dependent regulation of transcription in their requirement for a multicomponent RNA polymerase II complex.

The large subunit of RNA polymerase II contains a regulatory C-terminal domain (CTD), which consists of multiple heptad repeat repeats of the sequence YSPTSPS, which is the target for phosphorylation by cellular kinases (8, 9). The unphosphorylated form of RNA polymerase II, designated IIA, is exclusively found in preinitiation complexes, whereas the highly phosphorylated form of RNA polymerase II, designated IIB, is found in elongating transcription complexes (8, 9). It has been suggested that phosphorylation of the CTD contributes directly to promoter clearance and the processivity of RNA polymerase II (10–12). In addition, the CTD is required for the binding of suppressors of RNA polymerase II to form the RNA polymerase II holoenzyme complex that is critical for enhancer activity in vivo (13, 14). In this study, using different forms of RNA polymerase II that contained various numbers of the heptad repeat repeats, we studied the requirement of the CTD for trans-activation by Tat of human immunodeficiency virus (HIV) type 1 (HIV-1) and for the production of short, attenuated transcripts that are transcribed in the absence of Tat.

MATERIALS AND METHODS

Cell Culture and Transfection. COS cells were maintained in 10-cm dishes. They were transfected with Lipofectin (GIBCO/BRL) using 5 μg of reporter DNA, 5 μg of pSVTAT or pSVTATZX, and 10 μg of the indicated RPOI21S-CTD plasmids for 5 hr. After an additional 10 hr required for the expression of encoded proteins, a-amanitin was added to the medium (2.5 μg/ml) and the cells were incubated for an additional 48 hr (14). Trypan blue staining revealed no increased mortality of cells transfected with the a-amanitin-resistant RNA polymerases II containing only 31 or 5 CTD repeats (Δ31 and Δ5) compared with the wild-type RNA polymerase II after 60 hr of a-amanitin treatment. RNA for RNase protection experiments was prepared from the cytoplasmic fractions of transfected cells as described previously (15).

RNase Protection Assay. Twenty micrograms of RNA were used for the RNase protection assay. To make rabbit β-globin or HIV long terminal repeat (LTR) probe, Sp6BTS or pGEMI/WT vector was linearized with EcoRI and transcribed with SP6 RNA polymerase or T7 RNA polymerase to produce [α-32P]UTP-labeled RNA probe, respectively (15, 16). These assays were performed with the Guardian RNase protection kit (Clontech). Protected fragments were separated on 6% or 11% polyacrylamide/urea sequencing gels and exposed to x-ray film. Bands were quantified by the Image Analysis system (Alpha Innotech, San Leandro, CA). Background intensity was subtracted from the intensity of each band and relative intensity was calculated as the percentage of the intensity of each band relative to the most intense band in the lane on the gel.

Western Blots. Samples (20 μg) of whole-cell lysates were analyzed by Western blot analysis after SDS/15% polyacrylamide gel electrophoresis, electroblotted onto Immobilon-P membranes (Millipore) and probed with the α-hemagglutinin antibody 12CA5 (Boehringer Mannheim). Immune complexes were visualized with enhanced chemiluminescence (Amer sham).

In Vitro Transcription. In vitro transcription reactions were carried out using the HIV-1 promoter in the presence or absence of Tat, as described previously by Sheline et al. (17). Endogenous RNA polymerase II activity was inhibited by preincubating 50 μg of HeLa nuclear extract with 1.7 μg of mAb SWAG16 in TM/0.1 M KCl for 10 min at 4°C (TM = 50 mM Tris-HCl, pH 7.9/125 mM MgCl2/1 mM EDTA/20% glycerol/0.1 M phenylmethylsulfonyl fluoride/1 mM DTT). Reactions were supplemented with 1.4 μg (10 milliunits) of RNA polymerase IIA or RNA polymerase IIB, and incubated 10 min at 30°C prior to the addition of the DNA template, purified Tat protein (100 ng per reaction), and nucleotides.

Abbreviations: HIV, human immunodeficiency virus; LTR, long terminal repeat; TAR, trans-activation response element; CTD, C-terminal domain of RNA polymerase II; Tat, trans-activator; SV40, simian virus 40; HIVβglo, the HIV-1 LTR linked to the β-globin gene.

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The RNA polymerase IIB fraction had been preincubated with either 1.4 or 2.3 μg of mAb 8WG16 for 10 min at 4°C, as indicated in the legend of Fig. 5. Purified RNA polymerase IIA and RNA polymerase IIB were generously provided by Nancy Thompson and William Burgess (University of Wisconsin, Madison). The RNA polymerase IIA fraction was purified from calf thymus using immunoaffinity chromatography with mAb 8WG16, as described by Thompson et al. (18) and modified by Buermeyer et al. (19). RNA polymerase IIB was purified from calf thymus extracts following the protocol described by Hodo and Blatti (20).

RESULTS

The CTD of RNA Polymerase II Is Required for Tat Trans-Activation in Cells. The effect of the CTD on Tat trans-activation in vivo was tested by expressing different α-amanitin-resistant forms of RNA polymerase II, which contained various numbers of heptapeptide repeats (HA-WT, HA-Δ31, and HA-Δ5), encoding 52, 31, or 5 heptapeptide repeats, respectively, Fig. 1A), in COS cells. HIV promoter activity was monitored with a reporter plasmid in which the HIV-1 LTR was linked to the β-globin gene (HIVβglo, Fig. 1B), and HIVβglo transcription was measured by an RNase protection assay with an RNA probe complementary to the rabbit β-globin gene. As presented in Fig. 2, the wild-type RNA polymerase II (with 52 heptapeptide repeats) and the truncated CTD possessing 31 heptapeptide repeats both supported Tat-activated transcription (Fig. 2 Left, lanes 1 and 2). In sharp contrast, RNA polymerase II carrying only 5 heptapeptide repeats dramatically abolished effects of Tat (Fig. 2 Left, lane 3). As reported previously, transcription from 4XSpl (Fig. 1B) was unaffected by the truncation of the CTD (Fig. 2 Left) (14). Additionally, Tat had no effect on transcription from 4XSpl. Whereas the ratio of relative intensities of HIV-globin to the internal control of the sample with the wild-type RNA polymerase II or RNA polymerase II with 31 heptapeptide repeats was 1, that with the RNA polymerase II with 5 heptapeptide repeats was only 0.03. Equivalent amounts of Tat protein were expressed in these cells (Fig. 2 Upper Right). We conclude that transcriptional activation by Tat requires the CTD of RNA polymerase II in vivo.

Basal Transcription from the HIV-1 LTR Does Not Require the CTD of RNA Polymerase II in Cells. We next tested the requirement of the CTD for the basal transcription in the absence of Tat from the HIV-1 LTR in vivo. The wild-type RNA polymerase II and the truncated CTD possessing 31 heptapeptide repeats supported basal and Tat-activated transcription (Fig. 3). As presented in Fig. 2, RNA polymerase II carrying only 5 heptapeptide repeats dramatically abolished effects of Tat (Fig. 3 Upper Left, lane 3) but did not affect basal levels of transcription observed in the absence of Tat (Fig. 3 Upper Right, lane 7). This result mirrored the activity of the SV40 enhancer, which also required the CTD (Fig. 1B, SV40; Fig. 3 Lower Left, lane 3). However, the activity of 4XSpl was not affected by the truncation of the CTD (Fig. 3 Lower Right) (14). Faint bands shown in lane 4 or lane 8 of Fig. 3 Upper and Lower Left panels represent transcripts that have been made within 10 hr of incubation before the α-amanitin-containing medium was added. We conclude that the transcriptional
effects of Tat but not basal transcription from the HIV-1 LTR requires the CTD of RNA polymerase II in vivo. Thus, Tat trans-activation resembles the activity of transcriptional enhancers.

Promoter-Proximal (Attenuated) Transcription from the HIV-1 LTR Is Also Independent of the CTD of RNA Polymerase II. Whereas most viral transcripts terminate in the 5′ LTR and result in the accumulation of short, nonpolyadenylated transcripts of 55–59 nucleotides in the absence of Tat, more than 99% of transcripts are full-length in the presence of Tat, indicating that Tat principally affects rates of elongation rather than initiation of transcription (2). To examine the possibility that the CTD is required only for the formation of long (promoter-distal) transcripts and not for the attenuated (promoter-proximal) transcripts, we analyzed short and long transcripts from the HIV-1 LTR, using an RNA probe corresponding to TAR and U5 sequences. As presented in Fig. 4, full-length transcripts which formed in the presence of Tat were dramatically reduced with the shortest CTD (Fig. 4, lanes 1–4). Ratios of long transcripts to total transcripts [LT/(LT + ST)] were calculated by dividing the intensity of long transcripts by the sum of intensities of long and short transcripts. Whereas the percentages of long transcripts with the wild-type RNA polymerase II and RNA polymerase II with 31 heptapeptide repeats were 48 and 50, respectively, that with RNA polymerase II with 5 heptapeptide repeats was only 3. On the other hand, the formation of prematurely terminated transcripts was unaffected by the length of the CTD in the presence or absence of Tat (Fig. 4, lanes 1–8). LT/(LT + ST) of these bands were almost the same (2 or 3). Similar results were obtained using a stably transformed HeLa-Tat cell line, which expresses Tat constitutively (data not presented). These results indicate that whereas promoter-proximal transcription does not require the CTD, the activation of transcriptional elongation by Tat is absolutely dependent on the CTD.

Tat Trans-Activation in Vitro Also Requires the CTD of RNA Polymerase II. To assess further the role of the CTD in Tat trans-activation, in vitro transcription reactions were carried out using HeLa nuclear extracts in which the endogenous RNA polymerase II was inactivated by incubation with anti-CTD antibodies (mAb 8WG16) according to the procedure described by Buermeyer et al. (19). Reaction mixtures were then supplemented with purified fractions of wild-type RNA polymerase IIA or a form of RNA polymerase IIB lacking the CTD (16, 17). As presented in Fig. 5, the addition of the anti-CTD antibodies reduced both basal and Tat-activated transcription (compare lanes 3 and 4 with lanes 1 and 2). However, the addition of RNA polymerase IIA fully restored basal and Tat-activated transcription (Fig. 5, lanes 5 and 6), whereas the addition of RNA polymerase IIB (lacking the CTD) partially restored only basal transcription (Fig. 5, compare lane 7 with lanes 1 and 3) and did not promote Tat trans-activation (Fig. 5, compare lanes 8 and 4). Control reactions indicated that RNA polymerase IIA and RNA polymerase IIB were equally capable of activating basal transcription from the adenovirus major late promoter (Fig. 5, compare lanes 11 with 12 and 13), and similar results were obtained using the HIV-1 LTR at higher levels of DNA, where basal levels of transcription can be readily detected in the absence of Tat (data not presented). These results indicate that Tat trans-activation, but not basal HIV-1 transcription, depends on the CTD. As the CTD requirement could reflect the need for transcription factors that are bound to the CTD (13) or CTD bound kinases, we noted that neither basal nor Tat-activated transcription was inhibited by recombinant glutathione S-transferase-CTD protein, indicating that the CTD alone is not sufficient to bind factors necessary for effects of Tat (P. Wei and K.A.J., unpublished data). Taking these observations together with our in vivo data, we conclude that the increased processivity of
The CTD of RNA polymerase II is required for Tat trans-activation in vitro. In vitro transcription reactions were carried out with the HIV-1 LTR in the absence or presence of Tat, as indicated below the autoradiogram. Reactions in lanes 1 and 2 displayed basal and Tat-activated RNA levels, respectively, in the presence or absence of any anti-CTD antibody. Reactions presented in lanes 3–8 were preincubated with 1.7 μg of the anti-CTD antibodies to inactivate the endogenous RNA polymerase II. These reaction mixtures were then supplemented with either 1.4 μg (10 milliunits) of purified RNA polymerase IIA (lanes 5 and 6) or with an equivalent amount of purified RNA polymerase IIB (lanes 7 and 8). The arrow denotes specific HIV run-off transcripts (480 nucleotides). Whereas the reaction in lane 9 did not contain any anti-CTD antibody, reactions in lanes 10–13 were each preincubated with 1.7 μg of the anti-CTD antibody. The reaction in lane 11 was supplemented with 1.4 μg of purified RNA polymerase IIA and lanes 12 and 13 contained 1.4 μg of RNA polymerase IIB along with additional aliquots of 1.4 and 2.3 μg of the anti-CTD antibodies, respectively. The arrow denotes specific adenovirus major late promoter run-off transcripts (540 nucleotides).

RNA polymerase II by Tat requires the CTD of RNA polymerase II.

**DISCUSSION**

Our results indicate that the CTD of RNA polymerase II is required for effects of Tat but not for promoter-proximal transcription from the HIV-1 LTR both in vivo and in vitro. Thus, effects of Tat resemble those of previously described enhancer elements (14). They also reveal that initiation complexes can assemble on the viral promoter and transcribe TAR in the absence of the CTD. Thus, the RNA polymerase II with a severely truncated CTD is still capable of significant transcription on the HIV-1 LTR and the adenovirus major late promoter.

The CTD is a unique feature of RNA polymerase II, and it is not present in RNA polymerases I or III or the bacterial or viral RNA polymerases, although its function has not been elucidated completely (8, 9). The CTD has been proposed to stabilize preinitiation complexes by interacting with general transcription factors, including the TATA-binding protein, and it is also required for the association of suppressors of RNA polymerase II, general transcription factors, and other proteins in the RNA polymerase II holoenzyme complex (22, 23). Recently, we observed that Tat is part of the RNA polymerase II holoenzyme and that immobilized Tat can purify transcriptional complexes, which with addition of transcription factor IIB and TATA-binding protein support Tat trans-activation on the wild-type but not mutant TAR templates (T. P. Cuje and B.M.P., unpublished data).

Because the formation of preinitiation complexes requires the unphosphorylated CTD, whereas the CTD in elongating complexes is extensively phosphorylated, the CTD has also been reported to play a role in regulating the transition from initiation to elongation of transcription (8–12). Thus, our data suggest that Tat might affect transcriptional elongation by influencing the state of phosphorylation of the CTD. Indeed, Rice and colleagues have detected a Tat-associated kinase that is capable of phosphorylating the CTD in vitro, which might play a role in Tat trans-activation (24). An attractive model compatible with these data is that a single-step assembly of Tat and RNA polymerase II holoenzyme is followed by the activation of Tat at TAR leading to the phosphorylation of the CTD and subsequent transcriptional elongation. Additionally, Tat might remain associated with the elongating RNA polymerase II, and this association is also CTD dependent (25). However, the determination of the precise role of the CTD in Tat trans-activation must await the identification of relevant transcriptional co-activators of Tat.

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