Requirements for RNA Polymerase II Carboxyl-terminal Domain for Activated Transcription of Human Retroviruses Human T-Cell Lymphotropic Virus I and HIV-1*

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The carboxyl-terminal domain (CTD) of RNA polymerase (RNAP) II contains multiple repeats with a heptapeptide consensus: Tyr-Ser-Pro-Thr-Ser-Pro-Ser. It has been proposed that phosphorylation of this CTD facilitates clearance and elongation of transcription complexes initiated at the promoters. However, not all transcribed promoters require RNAP II with full-length CTD. Furthermore, different activators can promote capably the transcriptional activity of polymerase II mutants deleted in the CTD. Thus, the role of the RNAP II CTD in transcription and in response to activators remains incompletely understood. To study the role of CTD in the regulated transcription of human retroviruses human-T cell lymphotropic virus I and human immunodeficiency virus 1, we used an α-amanitin-resistant system developed previously (Gerber, H. P., Haggmann, M., Seipel, K., Georgiev, O., West, M. A., Littingung, Y., Schaffner, W., and Corden, J. L. (1995) Nature 374, 660–662). We found that transcription directed by the human T-cell lymphotropic virus I activator protein Tax was strongly promoted by CTD-deficient RNA polymerase II. By contrast, the human immunodeficiency virus 1 activator Tat, which is recruited to the promoter by tethering to a nascent leader RNA, requires CTD-containing polymerase II for transcriptional activity. Biochemically, we characterized that Tat associated with a cellular CTD kinase activity, whereas Tax did not. Concordantly, we found that cellular transcription factor Sp1, which can activate CTD-deficient polymerase II with an efficiency similar to Tax, also failed to bind a CTD kinase. Taken together, these observations address mechanistic corollaries between activators with(out) a linked CTD kinase and regulated transcription by RNA polymerase II moieties with(out) a CTD.

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‡The abbreviations used are: CTD, C-terminal domain; PAGE, polyacrylamide gel electrophoresis; pol, polymerase; RNAP, RNA polymerase; HIV, human immunodeficiency virus; HTLV, human T-cell lymphotropic virus; LTR, long terminal repeat; CAT, chloramphenicol acetyltransferase; GST, glutathione S-transferase; RPA, RNase protection assay; wt, wild type.
comparative paradigms for exploring the settings in which CTD-containing polymerases might be required for retroviral activity. Here, we have characterized the requirements for RNAP II CTD in the transcriptional functions of Tax and Tat.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—Plasmids expressing RNA polymerase II with various lengths of CTD were graciously provided by J. Corden (Johns Hopkins University) (1). Reporters containing chloramphenicol acetyltransferase (CAT) cDNA driven by the minimal HIV-1 LTR TATAA box with either six Sp1 sites or three Gal4 binding sites positioned upstream have been described (29). Trans-activator plasmids include Tat (28), Gal4-Tax (30), Gal4-Sp1A (31), Gal4-VP16 (32), Gal4-TBP1-56, and Gal4-CTD32x (33). GST-Tat, GST-Sp1A, and GST-CTD fusion constructs were constructed using pGEX2T (Pharmacia Biotech). GST-CTD was a gift from W. Dynan (University of Georgia) (34).

**RNase Protection Assays**—2 × 10^6 HeLa cells were plated in 10-cm dishes. On day 1, cells were transfected with 10 μg of polymerase II expression plasmid, 5 μg of CAT reporter, and 5 μg of trans-activator expression plasmid using Lipofectin (Life Technologies, Inc.). On day 2, plating media were replaced with media containing 3 μg/ml α-amanitin (Calbiochem). On day 3, cells were refed with fresh media containing α-amanitin. Approximately 48 h after the Lipofectin-DNA mixture was first introduced, total RNA was isolated using RNAzol B (Tel-Test, Friendswood, TX). Probes were prepared using a MaxiScript T7 in vitro transcription kit (Ambion, Austin, TX) and were gel-purified. 1 × 10^7 cpm/10 μg of probe was precipitated with total cellular RNA. Samples were processed using the RNase protection assay (RPA) II procedure as described by the manufacturer (Ambion). Briefly, RNA and probe were denatured at 95°C, hybridized overnight at 42°C, digested with RNase at 37°C for 30 min, precipitated with ethanol, and resolved in an 8% urea-polyacrylamide gel (Life Technologies). Signals were visualized by autoradiography and/or Fuji phosphorimaging.

**Transient Transfection for CAT Assays**—Three hours prior to transfection, HeLa cells were treated with either 10 nM staurosporine or mock treated with MeSO. 3 μg of reporter plasmid with 2 μg of trans-activator expression plasmid were then co-transfected by calcium phosphate precipitation. In the case in which Tat was expressed from the trans-activator plasmid, only 0.1 μg was used because of the high efficiency of this trans-activator. Cell lysates were assayed 48 h later for CAT activity; acetylations were quantitated by scintillation counting of silica gel slices.

**Protein Analysis**—HeLa cells treated either with 10 nM staurosporine or mock treated with MeSO overnight were pulse-labeled for 2 h with [32P]orthophosphate and [35S]methionine (ICN) or 1 μCi of [3H]methylmethanethiosulfonate (Amersham Corp.). Cells were lysed with radioimmunoprecipitation assay buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 8.0, and 1 mM phenylmethylsulfonylfluoride) and normalized for protein amounts (Bio-Rad). Equivalent amounts of protein were then electrophoresed into buffer (150 mM NaCl and 50 mM Tris-HCl, pH 8.0) and were immunoprecipitated overnight with 1 μg of anti-RNA polymerase II monoclonal antibody (8WG16; Promega) plus 15 μl of protein G-agarose (Oncogene Science, Cambridge, MA). Immunoprecipitates were washed five times (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, and 0.05% Tween 20), and samples were solubilized (125 mM Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, 2% β-mercaptoethanol, and 0.1% bromophenol blue) by boiling. After resolution of proteins by SDS-PAGE, the gel was visualized by Fuji phosphorimaging.

**In Vitro Kinase Assays**—GST fusion proteins were immobilized at 0.2 μg/ml onto glutathione-Sepharose beads (Pharmacia). 20 μl of GST fusion protein-bound resins were equilibrated with 100 μg of HeLa cell extract (pre pared with modifications of the method of Dignam et al. (35)) for at least 2 h. Unbound material was recovered as the flow-through fraction. Resins were then washed three times at 50 volumes with column buffer. This was followed by two additional 50-volume washes with kinase reaction buffer containing 50 mM Tris- HCl, pH 7.5, 5 mM MnCl2, and 5 mM dithiothreitol. To initiate the kinase reaction, 50 μl of kinase buffer supplemented with 200 ng of GST-CTD and 10 μCi of [γ-32P]ATP were added to either 20 μl of resin eluate or 20 μl of flow-through fraction and were incubated for 15 min at room temperature. The reaction was terminated by addition of SDS-PAGE sample buffer. Samples were resolved by 8% SDS-PAGE and were visualized by autoradiography. For immunoprecipitation of in vitro kinase reactions, the samples were diluted 100-fold with buffer A (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, and 0.05% Tween 20) and immunoprecipitated overnight with 3 μg of anti-RNA polymerase II monoclonal antibody (8WG16; Promega) plus 15 μl of protein G-agarose (Life Technologies). Immunoprecipitates were washed three times with buffer A. Samples were solubilized and resolved by 8% SDS-PAGE. The gel was fixed in 30% methanol and 10% acetic acid, dried, and visualized by autoradiography.

**RESULTS**

To assess the CTD requirements in transcriptional activation by HTLV-1 Tax and HIV-1 Tat, we used an approach developed by Corden and co-workers (1). The approach hinges on the efficient expression of α-amanitin-resistant mutants of the large subunit of RNA polymerase II. Versions of α-amanitin-resistant mutants have been constructed to have different numbers of CTD heptapeptides (Fig. 1A). Hence, α-amanitin treatment of cells transfected with plasmids that express mutant RNAP II results in the “poisoning” of the endogenous wild type RNAP II such that subsequent transcription in the cell depends on the exogenously expressed resistant mutant. For our studies, we tested α-amanitin-resistant polymerases that have 52, 31, or 5 copies of CTD heptapeptides (Fig. 1A). Hence, α-amanitin treatment of cells transfected with plasmids that express mutant RNAP II results in the “poisoning” of the endogenous wild type RNAP II such that subsequent transcription in the cell depends on the exogenously expressed resistant mutant. For our studies, we tested α-amanitin-resistant polymerases that have 52, 31, or 5 copies of CTD heptapeptides (Fig. 1A) for transcriptional responsiveness to co-introduced Tax- or Tat-expressing plasmids. Transcription directed by the RNA polymerase mutants was assayed by co-introduction into cells of a third plasmid as a reporter (either p6xSp1CAT or p-43GalCAT; Fig. 1B). p6xSp1CAT was used in assays for Tat activity. p-43GalCAT was used as the reporter for Gal-Tax and other Gal fusion protein-containing activators (Fig. 1C).
**FIG. 2. Determination by RNase protection assay of transcription directed by RNAP II mutants.**

**A.** Left panel, Tax-mediated activation is CTD length independent. A representative RPA analysis of Gal-Tax-activated transcription is shown. MWM, molecular size markers consisting of $^{32}$P end-labeled, MspI-restricted pBR322 DNA (lane 1). CAT (lanes 2 and 3) and actin (lanes 12 and 13) input probes are shown as either untreated (−) or treated with RNase (+). Plasmids used in the transfections are indicated at the top. Arrows, positions of the expected sizes of protected mRNAs for CAT or actin. Actin mRNA was used as normalization for equivalent amounts of total RNA used in each protection assay. Because of its abundance and stable half-life, the amount of actin mRNA detected represents primarily transcripts accumulated prior to the addition of α-amanitin. Right panel, a longer exposure of the portion of the gel (left), which contains the protected CAT mRNA signals. 

**B.** Basal expression of CAT reporters is independent of RNAP II CTD lengths. RPA signals of CAT mRNA expressed from the reporters in the absence of Trans-activators are shown. Panels shown represent 200-fold equivalent overexposure compared with the same samples shown in A. Relative times of exposure are 2 days with an intensifying screen for p6xSp1CAT and 6 days with an intensifying screen for p-43GalCAT. 

**C.** Activated expression by some Trans-activators depend on RNAP II CTD length. Tat, Gal-TBP17-56, and Gal-CTD32x show approximately 10-fold higher RPA signal for CAT mRNA transcribed by either CTDwt or CTDΔ31 compared with CTDΔ5. By contrast, Gal-Tax, Gal-Sp1A, and Gal-VP16 show essentially invariant levels of CAT mRNA expression by any of the three forms of polymerase. The slightly higher levels of signal from CTDΔ5 in the Gal-Tax and Gal-Sp1A samples were found not to be statistically significant on repetition. Relative times of exposure using a phosphorimaging plate are 6 h for Tat, 9 h for Gal-Tax, and 12 h for Gal-TBP17-56, Gal-Sp1A, Gal-VP16, and Gal-CTD32x.
The Capacity of each version of α-amanitin-resistant polymerase II to mediate basal and activator-dependent transcription was assessed using an RPA. A typical experiment is shown in Fig. 2A. In this instance, HeLa cells, first treated with α-amanitin, were then transfected with reporter (p-43GalCAT) plus a mutant polymerase-expressing plasmid (CTDwt, CTDΔ31, or CTDΔ5; Fig. 2A, lanes 5–7) or reporter plus mutant polymerase plus Gal-Tax plasmid (Fig. 2A, lanes 9–11). Expression from p-43GalCAT was measured for each sample by RPA for CAT mRNA (Fig. 2A, CAT, arrow). All samples were also probed identically for actin mRNA (Fig. 2A, lanes 14–21, actin, arrow) to normalize for input. Residual endogenous wild type polymerase II activity that might have escaped α-amanitin poisoning was assessed by including a co-transfection with pUC19 (which does not carry an α-amanitin-resistant polymerase) in each set of transfections (Fig. 2A, lanes 4 and 8). A darker exposure of the portion of the gel that contains protected mRNA signals from lanes 4–11 is shown (Fig. 2A, right panel).

From this type of analysis, which was replicated twice, we concluded that Gal-Tax activated transcription approximately 20-fold over basal, and that RNA polymerase II with an extremely shortened CTD tail (i.e. CTDΔ5; Fig. 2A, lane 11) was comparable to RNA polymerase II with wild type CTD (CTDwt; Fig. 2A, lane 9) and intermediate length CTD (CTDΔ31; Fig. 2A, lane 10) in supporting this activation.

The above findings suggest that HTLV-I Tax belongs to a class of transcriptional activators that has a CTD-independent phenotype. To define better this observation, we wished to compare Tax in parallel with HIV-1 Tat and other previously described activators (Gal-TBP17-56, Gal-Sp1A, Gal-VP16, and Gal-CTD32x, Fig. 2C). To do this we first verified how basal expression from the two reporters, p6xSp1CAT and p-43GalCAT (Fig. 1A), might depend on the length of the polymerase CTD (Fig. 2B). In α-amanitin-treated HeLa cells, co-transfection of p6xSp1CAT with a CTDwt, CTDΔ31, or CTDΔ5 plasmid showed identical amounts of mRNA as measured by RPA (Fig. 2B; panels shown are 200-fold-equivalent increased exposure over the comparable basal samples presented in Fig. 2A). An absence of signal from the co-transfection of pUC19 with p6xSp1CAT (Fig. 2B) confirmed that we were indeed measuring transcription mediated by each of the exogenously introduced mutant RNA polymerases. A set of assays performed in parallel with p-43GalCAT as the reporter (Fig. 2B) yielded a comparable pattern of signals. From multiple replications of such assays, we concluded that basal transcription from the reporters is insensitive to the length of the RNA polymerase II CTD.

We next compared activator-dependent transcription (Fig. 2C). For all activators, complete experiments were performed as shown in Fig. 2A. However, only the critical gel portions most relevant for signal analysis are presented in Fig. 2C. We observed that for Tat, Gal-TBP17-56, and Gal-CTD32x, relative signal intensity differences of approximately 10-fold existed between transcription supported by CTDΔ5 and that by CTDΔ31 or CTDwt (Fig. 2C). On the other hand, Gal-Tax, Gal-Sp1A, and Gal-VP16 exhibited invariant transcriptional activities from each of the three polymerases, independent of their CTD length (Fig. 2C). Hence, statistically indistinguishable levels of CAT mRNA were synthesized by each of the mutant polymerases in response to the latter three activators.

It has been suggested that phosphorylation of CTD, more than the absolute length of CTD per se, is the critical event influencing polymerase II clearance from the promoter (reviewed in Ref. 36). We queried whether divergent requirements for phosphorylated RNA polymerase II CTDs might be the underlying reason why Tax and Tat exhibit differing CTD length phenotypes with the above three mutant polymerases. If such is the case, then in the setting of wild type RNAP II-mediated transcription, CTD-independent activators should be less sensitive than CTD-dependent activators to inhibitors of CTD kinase. To test this, staurosporine, a previously described inhibitor of CTD kinase (37), was used to treat cells transiently transfected with the reporter plus Tat, Gal-Tax, or Gal-Sp1A. Expression in staurosporine-treated cells was compared with that in mock-treated cells (Fig. 3A) to assess for the relative effects of the CTD kinase inhibitor on activator function. In these settings, we found that Tat-dependent expression was reduced in staurosporine-treated cells to 20% of that in control cells. Gal-Tax showed a smaller 2-fold reduction, which is in part consistent with the fact that Tax is a phosphoprotein, and phosphorylation of Tax is influenced by staurosporine (38). Indeed, the activity of Gal-Sp1A, which has a CTD-independent phenotype like Tax, actually showed a slight, but statistically insignificant, rise with staurosporine treatment.

We verified that treatment with staurosporine did in fact affect RNAP II phosphorylation. Mock-treated and staurosporine-treated cells were radiolabeled in parallel with [32P]orthophosphate and [35S]methionine and [35S]cysteine (lanes 1 and 2). Equivalent amounts of protein from treated and untreated cells were used in immunoprecipitations with 1 μg of anti-RNA polymerase II monoclonal antibody and protein A+G-agarose. Immunoprecipitates were washed, solubilized in sample buffer, separated on SDS-PAGE, and visualized by phosphorimaging. Arrows, two forms of polymerase: pol IIα, hyperphosphorylated form; pol IIα, nonphosphorylated form. Note that staurosporine treatment did not affect the recovery by immunoprecipitation of [35S]-labeled RNAP II, which is predominantly of the IIα form (lanes 1 and 2). However, the total immunoprecipitate from staurosporine-treated cells has a relative paucity of the phosphorylated IIα form (compare lanes 3 and 4) after exposure for [32P]-labeled moieties.
phosphorylated species of RNAP II in treated cells to approximately 10% of that in mock-treated cells (Fig. 3B, lane 3). Thus, these results, taken together with the activity results in Fig. 3A, suggest that the Tat and Tax CTD length phenotypes might be a secondary reflection of the relative requirements by the different activators for interaction with phosphorylated and nonphosphorylated RNAPII CTDs.

Many CTD kinases have been described (34, 39–42). In most instances, it is not entirely clear how the CTD kinase interacts with RNAPII. One plausible hypothesis is that an activator requires phosphorylated CTD for function might carry CTD kinase activity to the promoter. Conversely, CTD-independent activators might not show this associative property. To test this hypothesis for Tax and Tat, we checked biochemically for the ability of either to bind CTD kinase activity. GST fusion “pull-down” protein chromatography using either Tax or Tat as bait was performed. Retained proteins were assayed in vitro for kinase activity.

We initially compared pull-downs from GST-Tat and GST-Tax to that from GST alone (Fig. 4A). In these experiments, HeLa cell extracts were equilibrated for at least 2 h with glutathione-Sepharose beads bound with either GST or GST fusion protein. After equilibration, each set of beads was washed extensively with 150 column volumes of buffer. We checked the last column wash to verify that it was free of CTD kinase activity (data not shown). We then assayed CTD kinase activity from beads eluted with 0.25 M KCl. For comparison, CTD kinase activity in the flow-throughs was assayed in parallel. Kinase reactions with GST-CTD supplied as substrate showed a distinctly phosphorylated moiety with the correct migration size in the reactions using the GST-Tat eluate (Fig. 4A, lane 4). In comparison, this band was absent from counterpart reactions from GST alone (Fig. 4A, lane 2) or GST-Tax (Fig. 4A, lane 6) beads. Under these binding conditions, we observed that virtually all of the input CTD kinase activity could be retained by GST-Tat (note the markedly diminished signal at the GST-CTD migration position in Fig. 4A, lane 3, flow-through), suggesting an efficient interaction between Tat and kinase.

We confirmed that the observed phosphorylated band was GST-CTD and not from phosphorylation of a protein contaminant that remained after purification. We immunoprecipitated each kinase reaction with the 8WG16 monoclonal antibody for RNAPII CTD (Fig. 4B). The phosphoprotein in the GST-Tat kinase fraction (Fig. 4A, lane 4) was indeed efficiently recovered (Fig. 4B, lane 5), confirming its identity as GST-CTD. Consistent with our interpretation, no phosphorylated GST-CTD was immunoprecipitated from either GST or GST-Tax fractions (Fig. 4B, lanes 4 and 6). We also checked the flow-through fractions (Fig. 4A, lanes 1, 3, and 5). Because these immunoprecipitations were performed on a larger, more diluted volume, longer exposures of gels were done, which verified the presence of phosphorylated GST-CTD (Fig. 4B, lanes 1–3). The fact that other phosphorylated moieties in the flow-throughs (Fig. 4A, lanes 1, 3, and 5) were not precipitated (Fig. 4B, lanes 1–3) supports the specificity of our immunoprecipitations.

The findings from Tax and Tat suggest a correlation between CTD independence and dependence and kinase association and lack of association. To determine whether this might also hold for a nonviral activator, we checked GST-Sp1A for associated CTD kinase activity. Because Sp1A has CTD-independent activity (Fig. 2C), one prediction is that it would not complex with a CTD kinase. In side-by-side comparisons with GST and GST-Tat, GST-Sp1A indeed failed to retain CTD kinase activity (Fig. 4C, lane 6).

**DISCUSSION**

Mechanisms governing transcription and activated transcription from polymerase II promoters hold many complex and incompletely resolved issues. Part of the complexity of pol II promoters is perhaps contributed by the inherent structure of the RNAPII molecule. Like RNAPI and RNAPIII, RNAPII
needs to enter and dock onto a core promoter complex to initiate transcription. However, unlike RNAPI or RNAPII, after entry and docking additional steps are required to facilitate the egress of RNAPII from the promoter. It has been suggested that the pol II CTD contributes to the recruitment, attachment, and (on modification) clearance of the polymerase from the promoter (reviewed in Ref. 36). In this regard, it is important to note that several investigators have shown that pol II CTD can interact directly with general transcription factor IID (43–47), that this interaction could impede departure of the polymerase from the promoter (48, 49), and that phosphorylation of CTD after attachment of polymerase to the nucleated transcription factor IID-IIB complex effects conformation changes influencing disengagement of pol II from the initiated ensemble (8, 9, 50).

The question is thus raised: how do activators affect clearance of RNAPII from the promoter? There are at least four distinct types of activation domains (reviewed in Ref. 51) with differences in mechanistic properties (1, 52). It is clear that many activators contribute directly to the assembly of RNAPII into the initiation complex at the promoter (reviewed in Ref. 36). However, because steady state transcription is a multicycle process in which productive disassembly of a previous cycle permits reassembly for the next cycle, it stands to reason that initiation events and clearance and elongation events at the promoter are intimately (and, in some instances, inseparably) linked (47, 53, 54). This concept has important implications for human retrovirus transcription, since it has been a standing paradox as to how HIV-1 Tat apparently influences initiation and elongation of RNAPII simultaneously (reviewed in Refs. 41 and 61). Our findings here that Tat influences polymerase II transcription in a CTD-dependent fashion that is linked to phosphorylation, together with genetic evidence to be presented elsewhere, suggest that this activator functions critically at the step of promoter clearance. Clearance of the polymerase in a prior round by Tat is then reflected in increased access and efficiency of initiation complex reassembly for a subsequent round of transcription (56).

Approximately six different CTD kinases that phosphorylate at serine, threonine, or tyrosine residues have been identified (34, 39–42, 55). A recently suggested yet unidentified Tat-associated kinase (57), which has activity on a CTD substrate, adds complexity to the picture. Physiologically, it is unclear why so many kinases impinge on a common substrate. However, it is clear that RNAPII functions in pleiotropic settings and contacts diverse activators and adaptors; each unique scenario might thus dictate a different activator-kinase-substrate interaction. Similarly, HIV-1 Tat is also known to have roles beyond a singularly focused activity at the promoter (58). In this regard, Tat has been shown previously to bind protein kinase R (59) which likely explains a Tat-dependent activation of nuclear factor xB (60, 64). Whether protein kinase R or a different kinase associates with Tat for promoter proximal kinase activity remains to be clarified.

It is intriguing that two related human retroviruses, HTLV-I and HIV-1, have evolved very different mechanisms for transcriptional regulation, although both conserve striking similarities in posttranscriptional regulation of mRNA metabolism (reviewed in Refs. 61 and 62). Conceivably, the differences in transcriptional activation are reflected by the extreme dissimilarities between the activation domains of Tax (30) and Tat (63) and the processes by which either is recruited to the promoter (see the Introduction). One can only speculate on whether these molecular differences contribute to the divergent pathogenic characteristics between the two viruses (i.e. HIV-1 is rapidly cytophilic, whereas HTLV-I is a transforming virus that has an extended cellular latency).
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