Protein Phosphatase-1 Dephosphorylates the C-terminal Domain of RNA Polymerase-II*

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Kareem Washington‡‡, Tatyana Ammosova‡, Monique Beullens§§, Marina Jerebtsova¶, Ajit Kumar,**, Mathieu Bollen§, and Sergei Nekhai‡‡‡

From the §Center for Sickle Cell Disease and Department of Biochemistry and Molecular Biology and ¶Program in Genetics, Howard University, Washington, D. C. 20059, the **Division of Biochemistry, Catholic University of Leuven, B-3000 Leuven, Belgium, †Children’s National Medical Center, Washington, D. C. 20010, and the ‡‡Department of Biochemistry and Molecular Biology, George Washington University, Washington, D. C. 20037

Transcription by RNA polymerase-II (RNAPII) is controlled by multisite phosphorylation of the hexapeptide repeats in the C-terminal domain (CTD) of the largest subunit. Phosphorylation of CTD is mediated by the cyclin-dependent protein kinases Cdk7 and Cdk9, whereas protein serine/threonine phosphatase FCP1 dephosphorylates CTD. We have recently reported that human immunodeficiency virus-1 (HIV-1) transcription is positively regulated by protein phosphatase-1 (PP1) and that PP1 dephosphorylates recombinant CTD. Here, we provide further evidence that PP1 can dephosphorylate RNAPII CTD. In vitro, PP1 dephosphorylated recombinant CTD as well as purified RNAPII CTD. HEK nuclear extracts were found to contain a species of PP1 that dephosphorylates both serine 2 and serine 5 of the heptapeptide repeats. In nuclear extracts, PP1 and FCP1 contributed roughly equally to the dephosphorylation of serine 2. PP1 co-purified with RNAPII by gel filtration and associated with RNAPII on immunoaffinity columns prepared with anti-CTD antibodies. In cultured cells treated with CTD kinase inhibitors, the dephosphorylation of RNAPII on serine 2 was inhibited by 45% by preincubation with okadaic acid, which inhibits phosphatases of the PPP family, including PP1 but not FCP1. Our results demonstrate that RNAPII CTD is dephosphorylated by PP1 in vitro and by PPP-type phosphatase, distinct from FCP1, in vivo.

Transcription of eukaryotic protein-encoding genes is mediated by RNA polymerase II (RNAPII). During a single cycle of transcription, the largest subunit of RNAPII is extensively phosphorylated and then dephosphorylated on its C-terminal domain (CTD) containing a tandem array of 26–52 heptapeptide repeats with the consensus sequence Tyr<sup>1</sup>-Ser<sup>2</sup>-Pro<sup>3</sup>-Thr<sup>4</sup>. Ser<sup>5</sup>-Pro<sup>6</sup>-Ser<sup>7</sup> (1). The hypophosphorylated RNAPII (RNAPIIa) is recruited to the preinitiation complex, in which RNAPII is hyperphosphorylated (RNAPII<sub>o</sub>) by the cyclin-dependent kinase Cdk7, a subunit of the general transcription factor IIA (2). During elongation of transcription, the CTD is additionally phosphorylated by Cdk9 (3). Phosphorylation of the CTD is required for promoter cleavage, efficient transcription elongation, and recruitment of mRNA processing factors, including capping enzyme and splicing factors (4).

At the end of the transcription cycle, RNAPII is dephosphorylated by a phosphatase, FCP1, which is the only known member of a novel family of protein phosphatases (5). FCP1 is required for the regeneration of initiation-competent RNAPII and may also antagonize the action of Cdk8, which inhibits transcription by phosphorylation of RNAPII prior to its recruitment to the preinitiation complex (6). FCP1 dephosphorylates Ser-2 during elongation of transcription but not Ser-5 during initiation of transcription (3). Therefore, it is likely that another, yet unidentified protein phosphatase is involved in regulation of CTD phosphorylation during transcription. In accordance with this view, okadaic acid, which inhibits phosphatases of the PPP family, including PP1, but not FCP1 (7), has been reported to induce RNAPII phosphorylation in growing HEK cells (8). Therefore, it is possible that a PPP-type protein phosphatase may dephosphorylate RNAPII CTD. Our previous studies have demonstrated that protein Ser/Thr phosphatase-1 (PP1) is involved in the regulation of HIV-1 transcription (9, 10). We have shown that HIV-1 Tat associates with PP1 (9) and that a Tat-associated CTD kinase protein complex contains a nuclear regulator of PP1, known as NIPPI (10). In our experiments, addition of purified PP1 induced the elongation of transcription from the HIV-1 promoter in vitro (9, 10). We have also reported that PP1 dephosphorylates recombinant CTD phosphorylated by CDK9/cyclin T1 (9). Taken together, our results suggested that PP1 might be implicated in the regulation of HIV-1 transcription by controlling the level of RNAPII phosphorylation.

PP1 belongs to the PPP family of serine-threonine protein phosphatases, which also includes PP2A, PP2B, and PP4-6 (11). With the exception of PP2B, the concentration of protein phosphatases of the PPP family is 2–10-fold higher in the nucleus than in the cytoplasm (11). The nucleus of mammalian cells contains multiple species of PP1. They consist of a constant catalytic subunit and a variable regulatory (R-) subunit that determines the localization, activity, and substrate specificity of the phosphatase (11). One of the major nuclear sub-
units of PP1 is NIPP1 (11). NIPP1 targets PP1 to nuclear speckles that also contain RNAPII (12). Therefore, it was of particular interest to analyze whether PP1 associates with RNAPII and/or mediates RNAPII dephosphorylation.

In the present study, recombinant CTD was hyperphosphorylated with purified Cdk7 (10) and used as a substrate for purified catalytic subunit of PP1 (PP1c). In addition, we also used as a substrate the entire RNAPII CTD, which was phosphorylated during transcription in vitro. Furthermore, a species of PP1 present in HeLa cell nuclear extracts was utilized to dephosphorylate the RNAPII CTD. We evaluated the relative contribution of PP1 and FCP1 to the dephosphorylation of Ser-2 in HeLa nuclear extracts using reported PP1 and FCP1 inhibitors. Next, we analyzed whether PP1 was present in the same macromolecular complex with RNAPII and whether there was a direct association between PP1 and RNAPII. Finally, we used the phenomena of rapid RNAPII dephosphorylation in the cells treated with Cdk7 and Cdk9 inhibitors (13) to analyze whether it was PP1 that mediated the RNAPII dephosphorylation. We used okadaic acid, a well established cell-permeable inhibitor of PP1 and PP2A (7), to test whether inhibition of protein phosphatases of the PPP family would prevent RNAPII dephosphorylation. Our results indicate that RNAPII dephosphorylation is mediated by PP1 in vitro and by PPP-like protein phosphatase in vivo.

EXPERIMENTAL PROCEDURES

Materials—COS-7 cells and HeLa cells were purchased from ATCC (Manassas, VA). Superose™ 6 HR 10/30 was obtained from Amersham Biosciences. The GST-fused CTD of RNAPII was expressed in *Escherichia coli* and purified as described (14). HeLa nuclear extract was prepared according to Ref. 15. Purified PP2A was purchased from Upstate Biotechnology (Lake Placid, NY). Phosphorylase-b was from Calzyme (Lake Placid, NY). Antibodies against the -subunit of the translation initiation factor eIF-2 (eIF-2α) were purchased from Sigma. Phosphopeptide-specific monoclonal antibodies SWG16 (specific for the unphosphorylated form of CTD), HS (CTD phosphorylated on Ser-2), and H14 (CTD phosphorylated on Ser-5) were all purchased from Berkeley Antibody (Richmond, CA). Antibodies against the α-subunit of the translation initiation factor eIF-2 (eIF-2α) were generated as described (17). Trypsin (T-8842), trypsin inhibitor (T-9003), okadaic acid, and DRB (6,8-dichloro-1-b-o-rifoborusanylbenzimidazole) were purchased from Sigma. Flavopiridol (cis-2-(2-chlorophenyl)-5,7-dihydroxy-8-[4-(3-hydroxy-1-methyl)piperidinyl]-4H-1-benzopyran-3-one hydrochloride) was a gift from Dr. Brady (NCI, National Institutes of Health, Bethesda, MD).

Preparation of Recombinant Proteins—Polyhistidine-tagged NIPP1 fragments and NIPP1 mutants were prepared as described previously (12, 18). HIV-1 Tat was expressed in *E. coli* and purified on Aquapore RP-300 column (Applied Biosystems, Foster City, CA) by reverse-phase chromatography as described (19).

Preparation of Phosphorylase-a and Dephosphorylation Assay—10 mg of phosphorylase-b was dissolved in 300 μl of buffer A (10 mM glycophosphate, pH 7.4, 50 mM 2-mercaptoethanol) and dialyzed against the same buffer for 2–3 h. Then 7.5 μl of 500 mM Tris-HCl (pH 8.0) and 6 μl of phosphorylase kinase prepared according to Ref. 20 were added and incubated for 10 min at 30 °C followed by addition of 45 μl of ATP-Mg mix (8.3 mM ATP, 83 mM MgCl₂, 75 mM Tris-HCl, 75 μl of γ-32P[ATP] (20 μM mCi), and 1 μl of RNAPII) and incubation for 2 h at 30 °C. Phosphorylase-a was precipitated with ammonium sulfate, resuspended in buffer A, and dialyzed against buffer A for 1–2 days at 4 °C. AG 50W-x8 (mixed anion and cation exchange) resin was placed in a separate dialysis bag to improve removal of unincorporated ATP and inorganic phosphate. Dialyzed phosphophorlase-a was dialyzed against 4°C. Approximately 0.2 nmol of phosphophorlase-a was used as a substrate for PP1 or PP2A. The phosphorylase phosphatase assay was carried out for 10 min in a buffer containing 50 mM glycglycine at pH 7.4, 0.5 mM dithiothreitol, and 5 mM β-mercaptoethanol as described (21). When indicated, prior to the phosphorylase phosphatase assay, the samples were trypsinized to generate free, non-catalytatic subunit of PP1 (21).

Preparation of Hyperphosphorylated CTD (CTD50 and Dephosphorylation Assay—Recombinant CTD was phosphorylated as we described earlier (22). Briefly, GST-CTD (100 ng) was phosphorylated in a 20-μl reaction with 50 μM ATP (1 μCi of [γ-32P]ATP) in kinase buffer (50 mM HEPES (pH 7.9), 10 mM MgCl₂, 6 mM EGTA, and 2.5 mM dithiothreitol) for 1 h at 30 °C. To induce CTD hyperphosphorylation, 40 μM mC2p peptide (ARAPGVVFTYAHERVTLLWRA (24)) was added to the kinase reaction. A randomized peptide rC2p (HARTVGVWYRAEFVTPAVV (24)) was used as a control. The reaction was supplemented with 7 μM EDTA to inactivate the kinase followed by addition of PP1 and incubation for 30 min at 30 °C. The reactions were resolved on 10% SDS-PAGE and analyzed with a phosphorimaging device (Packard Instrument Co.).

Phospho-Acid Analysis—The analysis was carried out on the Hunter thin-layer peptide mapping electrophoresis system (C.B.S. Scientific, Del Mar, CA) according to the manufacturer’s recommendations. Briefly, hyperphosphorylated CTD, prepared as described above, was resolved on 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane (Amersham Biosciences). The portion of the membrane containing CTDs was boiled in 5.7 μl HCl at 110 °C in order to liberate the phosphorylated phosphoamino acids as described (25). After hydrolysis, the sample was lyophilized and resuspended in the buffer for pH 1.9 electrophoresis with the addition of 0.06 mg/ml phosphoamino acid standards. The sample was resolved by thin-layer electrophoresis on a cellulose plate (C.B.S. Scientific) at pH 1.9 in the first direction and at pH 3.5 in the second direction. Cold standards were visualized by staining the plate with 0.25% ninhydrine dissolved in acetone. Positions of phosphorylated phosphoamino acids were analyzed with a phosphorimaging device (Packard Instrument Co.).

Dephosphorylation of RNAPII CTD—RNAPII was phosphorylated in transcription reaction (20 μl) containing 50 μg of HeLa nuclear extract; 0.5 mM ATP, CTP, UTP, and GTP; and 0.2 μg of JK2 linearized template (22) in transcription buffer (20 m M HEPES, pH 7.9, 50 mM KCl, 6.25 mM MgCl₂, 0.5 mM EDTA, 2 mM dithiothreitol, and 10% glycerol). Transcription was carried out for 1 h at 30 °C. Reaction was terminated by addition of 7 mM EDTA. RNAPII was immunoprecipitated with α-CTD (SWG16) antibodies coupled to protein A-agarose for 2 h at 4 °C and then extensively washed with TBS (20 mM Tris-HCl, pH 7.5, 137 mM NaCl). Alternatively, transcription reaction was terminated by 7 mM EDTA or flavopiridol and further incubated for 30 min at 30 °C to allow dephosphorylation of RNAPII by CTD phosphatases present in the nuclear extracts.

Size Exclusion Chromatography—HeLa nuclear extract (500 μg) was fractionated on Superose™ 6 HR 10/30 (Amersham Biosciences) in Buffer B (40 mM HEPES-KOH (pH 7.9), 100 KCl, 0.1 mM EDTA, and 1 mM dithiothreitol, 10% glycerol) containing 0.1% Triton X-100. Fractions (0.5 ml) were collected, concentrated on Microcon YM-10 spin columns (Millipore, Temecula, CA), and analyzed by SDS-PAGE.

Immunoinaffinity chromatography—The monoclonal α-CTD (SWG16) antibodies (1.5 mg) were coupled to 0.5 ml of N-hydroxy succinimide-activated Sepharose 4 Fast Blue (Amersham Biosciences). The coupled beads were washed and equilibrated with TBS (20 mM Tris-HCl, pH 7.5, 137 mM NaCl). HeLa nuclear extract (750 μg) was loaded on the column. After washing off-bound proteins with TBS, RNAPII was eluted with 10 mM sodium acetate (pH 3.4).

Analysis of RNAPII phosphorylation in cultured cells treated with kinase and phosphatase inhibitors—COS-7 cells, HEK 293 cells, and HeLa cells were cultured in 24-well plates until 75% of confluence in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% (v/v) fetal bovine serum. DRB was dissolved in 50% ethanol. Flavopiridol was dissolved in water. DBR (100 μM) or flavopiridol (25 μM) was added to the cell cultures for 1 h. Okadaic acid (dissolved in ethanol) was added as indicated at either 10 nM or 1 μM concentration for 30 min prior to the addition of DBR or flavopiridol. After the treatment, cells were washed in phosphate-buffered saline and lysed on the plates in SDS-PAGE loading buffer containing 2% SDS (100 μl per well). To estimate protein concentration, a portion of each lysate was briefly run on a 15% SDS-PAGE, allowing the samples to migrate for about 5 min into the resolving gel. The gel was stained with Coomassie Blue and photographed with a digital camera (Invitrogen), and quantified with phosphorimaging software. Lysates were resolved on 4% SDS-PAGE, transferred to polyvinylidene difluoride membrane, and analyzed by immunoblotting with monoclonal antibodies against non-phosphorylated, Ser-2, and Ser-5 phosphorylated RNAPII CTD.


Fig. 1. Preparation of CTDo. In A, the mC2p peptide stimulates GST-CTD hyperphosphorylation in a dose-dependent manner. Lanes 2–5, recombinant GST-CTD was incubated with purified Cdk7 in the presence of 5, 10, 20, and 40 μM mC2p peptide. Lane 1, control without mC2p peptide. Kinase reactions were resolved on 10% SDS-PAGE and analyzed by a phosphorimaging device. Addition of the peptide resulted in the appearance of the hyperphosphorylated form of CTD, indicated as CTDo. The hypophosphorylated form of CTD is indicated as CTDa. B, kinase reactions from panel A analyzed with OptiQuant phosphorimaging software. Quantification of the phosphorylated CTD is shown in arbitrary units, proportional to the phosphorimaging device units. Positions of hyper- and hypophosphorylated CTD are indicated as CTDo and CTDa.

RESULTS

Recombinant GST-CTD Is a Substrate for PP1c—We have recently reported that HIV-1 Tat and peptides derived from HIV-1 Tat enhanced the phosphorylation of RNAPII CTD by Cdk7/Cyclin H, Cdk2/Cyclin E, and Cdk9/Cyclin T1 (10, 23). In the absence of the peptide activators, recombinant GST-CTD was poorly phosphorylated by any of the kinases (10, 23). While analyzing the effect of Tat on CTD phosphorylation, we found that a peptide, a pseudosubstrate for Cdk7 (mC2p (24)), also stimulated CTD phosphorylation by purified Cdk7/cyclin H (Fig. 1), Cdk1/cyclin B, and Cdk2/Cyclin E (not shown). Addition of mC2p stimulated GST-CTD hyperphosphorylation in a concentration-dependent manner (Fig. 1A, lanes 2–5). Addition of mC2p increased the amount of CTDo more than 10-fold (Fig. 1B), which appears as an apparent higher molecular weight band on SDS-PAGE. On the other hand, CTDa, which appears as an apparent lower molecular weight band on SDS-PAGE (Fig. 1A), co-migrated with the non-phosphorylated CTD (Fig. 1E). A randomized peptide (rC2p) did not stimulate CTD phosphorylation by Cdk7/cyclin H (Fig. 1C, lane 3). The mC2p peptide also stimulated phosphorylation of purified yeast RNAPII (not shown). Phosphoamino acid analysis showed the presence of phosphoserines in CTDo (Fig. 1D). In contrast, histone H1 phosphorylated by Cdk7/cyclin H contained phosphorylated serines and threonines (data not shown). The phosphorylation level of RNAPII was visualized by Western blot analysis with phosphoepitope-specific monoclonal antibodies that recognized phospho Ser-2 (H5) or phospho Ser-5 (H14) in the heptapeptide repeats of the CTD. Immunoblotting analysis showed that Cdk7/cyclin H phosphorylated CTD in the presence of mC2p on Ser-2 and Ser-5 (Fig. 1E).

We utilized the effect of CTD hyperphosphorylation in the presence of mC2p to generate CTDo, which was used as a substrate for the catalytic subunits of PP1 and PP2A (Fig. 2). Although PP1 dephosphorylated CTDo in a concentration-dependent manner (Fig. 2, A and B, lanes 2–4), no dephosphorylation was seen with the structurally related PP2A (Fig. 2, D and E, lanes 2–4). Yet based on their activities toward the reference substrate, glycogen phosphorylase-a, PP2A was added at a 2-fold higher activity than PP1, dephosphorylating twice as much substrate as PP1 (Fig. 2, C and F, lanes 2–4). Moreover, since the specific phosphorylase phosphatase activity of PP2A is about 10-fold lower than that of PP1 (26), these data indicated that PP1 is at least a 20-fold better CTD phosphatase than is PP2A. The dephosphorylation of the CTD domain by 160 nM PP1 was completely blocked by the addition of 1 μM NIP (Fig. 3A, lanes 2 and 3), a specific inhibitor of PP1 (27). This observation provides evidence that dephosphorylation of CTDo by PP1 was not mediated by a contaminating phosphatase and that the dephosphorylation was not an artifact resulting from proteolysis of the CTD. Because a high concentration of mC2p was present during the dephosphorylation reaction (typically between 20 and 30 μM), we have also asked whether the peptide affected the protein phosphatase activity of PP1 or PP2A. We found that the addition of 30 μM of
is not likely to be FCP1 because EDTA inhibits the Mg\textsuperscript{2+} phatase (Fig. 4).

Endogenous PP1 Dephosphorylates RNAPII CTD—We subsequently examined whether the CTD domain of RNAPII could be dephosphorylated by the endogenous pool of PP1 present in HeLa cell nuclear extracts. Following phosphorylation of RNAPII during incubation of the extracts under conditions of transcription, the CTD kinases were blocked by 7 mM EDTA. We found that the addition of EDTA enabled the dephosphorylation of both Ser-2 and Ser-5 by an endogenous protein phosphatase (Fig. 4, lane 2). This endogenous protein phosphatase is not likely to be FCP1 because EDTA inhibits the Mg\textsuperscript{2+}-dependent FCP1 and PPM families (28). We identified the endogenous phosphatase as PP1 because the dephosphorylation of Ser-2 and Ser-5 was blocked by the addition of NIPP1 (Fig. 4, lane 3).

Relative Contributions of PP1 and FCP1 in the Dephosphorylation of Ser-2 Phosphorylated RNAPII CTD—FCP1 is a well-established CTD phosphatase that predominantly recognizes phosphorylated Ser-2 of CTD (3, 5). Therefore, it was of interest to estimate directly the contribution of FCP1 to the dephosphorylation of Ser-2. First, we analyzed the effect of inhibition of PP1 on the level of Ser-2 phosphorylation when FCP1 was not blocked by EDTA. Inhibition of PP1 with NIPP1 or okadaic acid in a transcription assay increased the level of Ser-2 phosphorylation by 30–45% (Fig. 5A, lanes 1–3). To estimate directly the contribution of FCP1 to the dephosphorylation of Ser-2, we made use of the reports that HIV-1 Tat inhibits FCP1 (29) but does not affect CTD dephosphorylation by PP1 (9). We also included a high concentration of flavopiridol to inhibit the CTD kinases (13) and to enable the dephosphorylation of RNAPII CTD by endogenous protein phosphatases (Fig. 5B, lane 2). We arbitrarily set the difference in Ser-2 phosphorylation in the reaction before and after addition of flavopiridol as a 100% (Fig. 5B, lanes 1 and 2). When PP1 was inhibited by NIPP1, and when FCP1 was active, ~40% of Ser-2 was dephosphorylated (Fig. 5B, lane 3). When FCP1 was inhibited by Tat, and when PP1 was active, ~30% of Ser-2 was dephosphorylated (Fig. 5B, lane 4). Therefore, PP1 dephosphorylates 30–45% of Ser-2 based on the observation in Fig. 5A and ~30% of Ser-2 based on the observations in Fig. 5B. Combining these results together, PP1 may dephosphorylate ~40% of Serine-2. Therefore, PP1 and FCP1 contributed roughly equally to dephosphorylation of Ser-2.

Co-purification of PP1 and RNAPII—PP1 is targeted to multiple organelles and macromolecular complexes by a host of regulatory subunits (11). We have examined whether PP1 is...
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A. PP1 and FCP1 Dephosphorylate Ser-2 of RNAPII. A HeLa cell nuclear extract was subjected to in vitro transcription for 1 h and then further incubated for 30 min without (lane 1) or with the addition of 5 μM NIPP1 (lane 2) or 100 nM okadaic acid (OA, lane 3). The phosphorylation level of serine 2 was quantified with phosphoepitope-specific antibodies. The blot was scanned and quantified with phosphoimaging software.

B. A HeLa cell nuclear extract was subjected to in vitro transcription (lane 1). Subsequently, the extract was treated with 25 μM flavopiridol (flavo) and further incubated for 30 min to enable RNAPII dephosphorylation without (lane 2) or with the addition of 5 μM NIPP1 (lane 3) or 20 μM Tat (lane 4). The phosphorylation level of serine 2 was quantified with phosphoepitope-specific antibodies. The blot was scanned and quantified with phosphoimaging software.

Fig. 5. PP1 and FCP1 Dephosphorylate Ser-2 of RNAPII. A, inhibition of PP1 increased the phosphorylation of RNAPII during in vitro transcription. A HeLa cell nuclear extract was subjected to in vitro transcription for 1 h and then further incubated for 30 min without (lane 1) or with the addition of 5 μM NIPP1 (lane 2) or 100 nM okadaic acid (OA, lane 3). The phosphorylation level of serine 2 was quantified with phosphoepitope-specific antibodies. The blot was scanned and quantified with phosphoimaging software. B, RNAPII dephosphorylation by PP1 and FCP1 in nuclear extracts. A HeLa cell nuclear extract was subjected to in vitro transcription (lane 1). Subsequently, the extract was treated with 25 μM flavopiridol (flavo) and further incubated for 30 min to enable RNAPII dephosphorylation without (lane 2) or with the addition of 5 μM NIPP1 (lane 3) or 20 μM Tat (lane 4). The phosphorylation level of serine 2 was quantified with phosphoepitope-specific antibodies. The blot was scanned and quantified with phosphoimaging software.

A PPP-type Protein Phosphatase Regulates RNAPII Dephosphorylation in Growing Cells—In growing cells, RNAPII is rapidly dephosphorylated when the cells are treated with inhibitors of Cdk7 and Cdk9 (DRB and flavopiridol, (5, 13)). It has also been reported that treatment of growing cells with 1 μM okadaic acid increases RNAPII phosphorylation (8). We utilized the effect of induced dephosphorylation of RNAPII and the effect of okadaic acid to analyze whether the dephosphorylation was in part mediated by a protein phosphatase of the PPP family. In growing COS-7 cells, RNAPII was phosphorylated on Ser-2 and Ser-5 (RNAPII, Fig. 7, A and B, lane 3). Treatment with flavopiridol (Fig. 7, A and B, lane 6) or DRB (not shown) resulted in a complete dephosphorylation of Ser-2 and Ser-5 residues. However, when the cells were pretreated with 1 μM okadaic acid prior to the treatment with flavopiridol, the dephosphorylation occurred only partially (Fig. 7A, lane 8). The pretreatment with okadaic acid also increased the RNAPII level (Fig. 7C, lane 8). Interestingly, the addition of 10 mM okadaic acid, which inhibits PP2A completely but has only a marginal effect on PP1 (7), had a much less pronounced effect on the recovery of Ser-2 and Ser-5 phosphorylation (Fig. 7, A and B, lane 7) and did not increase RNAPII level (Fig. 7C, lane 7). Similar results were obtained with HeLa cells (not shown).
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DISCUSSION

The present study describes a novel function of PP1, which is the dephosphorylation of RNAPII CTD. We provide evidence that PP1 dephosphorylates RNAPII in vitro and probably also in vivo. It is becoming clear that CTD phosphorylation is a dynamic process and is tightly regulated during transcription. Both protein kinases and protein phosphatases are of equal importance in the cycles of phosphorylation and dephosphorylation. The role of protein kinases has been widely demonstrated and reviewed (2), but only one RNAPII CTD phosphatase, FCP1, has been characterized (5). It has been shown that FCP1, which is part of RNAPII holoenzyme, dephosphorylates RNAPII CTD during elongation of transcription (3). Previous studies have shown that FCP1 does not recognize recombinant CTD or purified RNAPII as a substrate and requires the RNAPII holoenzyme for its phosphatase activity (28). However, in a recent report, FCP1 dephosphorylated a CTD fragment containing four hepta repeats in vitro at acidic pH (30).

The potential problem of the CTD dephosphorylation assay with full-length CTD is inefficient hyperphosphorylation of CTD by CTD kinases in vitro. Because some proteins such as HIV-1 Tat protein and its peptides could activate CTD kinases (10, 23), we hypothesized that kinase activation by peptides may be a useful approach for phosphatase studies. We generated CTDo substrate utilizing mC2p peptide. The mC2p peptide has been described by Cujec et al. (24). It contains 20 amino acids from the Cdk2 sequence flanking a threonine mutated to alanine at position 160, which is normally phosphorylated by Cdk1/cyclin H. The mC2p blocks Tat transactivation in vitro and in vivo (24, 31). Surprisingly, in our experimental conditions, mC2p increased the activities of Cdk1/cyclin H, Cdk2/cyclin E, and Cdk1/cyclin B when the recombinant CTD domain (23) or purified yeast RNAPII (not shown) were used as substrates. Activation of Cdk2 is a two-step dynamic process; the first step is a rapid association of Cdk with a cyclin and involves the PSEAIIE motif, and the second step is an interaction between the T-loop of Cdk and the N-terminal helix of a cyclin (32). Recognition of T-loop by a cyclin is regulated by protein-protein interactions outside the T-loop and is not dependent on the primary sequence of the T-loop (33). We speculate that the T-loop-derived peptide added at high concentration may interact with the cyclin and stimulate conformation changes needed for rearrangements of the ATP binding site (32).

Based on our recent finding that PP1 positively regulates HIV-1 Tat activated transcription, we hypothesized that PP1 may function as a CTD phosphatase. This view is in accordance with an earlier report showing that a treatment of HeLa cells with okadaic acid results in a hyperphosphorylation of RNAPIII (8). Two protein phosphatases of the PPP family have been used in our study, PP1 and PP2A. The phosphatases have a high degree of homology, and both are present in the nucleus (11). Therefore, they may be potentially involved in regulation of transcription. We showed here that PP1 but not PP2A dephosphorylated recombinant CTDo. To rule out the possibility that the loss of the hyperphosphorylated form of CTD is the result of proteolysis rather than dephosphorylation, we have utilized a well known specific PP1 inhibitor NIPP1 (27) and have shown that it blocks CTDo dephosphorylation.

The function of PP1 as a CTD phosphatase was confirmed using immunopurified RNAPII. PP1 effectively dephosphorylated RNAPII. Recently it was shown that recombinant FCP1 preferentially dephosphorylates Ser-2 of RNAPII over Ser-5 in vitro (30). We show here that PP1 efficiently dephosphorylated both Ser-2 and Ser-5 of immunopurified RNAPII.

Next, we analyzed whether HeLa nuclear extract contains a species of PP1, which may dephosphorylate RNAPII CTD. RNAPII was hyperphosphorylated during transcription assay, and then the kinases present in the nuclear extract were blocked by addition of 7 mM EDTA. Under these conditions, we observed spontaneous dephosphorylation of both Ser-2 and Ser-5 of CTD. Addition of EDTA blocks not only kinases but also the Mg2+-dependent FCP1 (28). Therefore, RNAPII dephosphorylation was mediated by a species of the PPP-type phosphatase. Because the RNAPII dephosphorylation was blocked by the addition of NIPP1, we conclude it was mediated by PP1 rather than by other phosphatases of the PPP subfamily.

To investigate the relative contribution of PP1 and FCP1 to CTD dephosphorylation in HeLa nuclear extracts, we specifically inhibited FCP1 or PP1 by the addition of HIV-1 Tat or NIPP1, respectively. Our analysis indicates that PP1 and FCP1 roughly contribute equally to the dephosphorylation of Ser-2 phosphorylated CTD. PP1 lacks a putative nuclear localization signal and is targeted to various subnuclear structures by specific targeting subunits (11). To assess the ability of PP1 to form a complex with RNAPII in the nucleus, we fractionated a HeLa nuclear extract by two different types of columns. We found that PP1 and RNAPII were co-migrated during gel filtration. Moreover, immunoaffinity chromatography, performed on a column prepared with immobilized anti-RNAPII antibodies, showed that a portion of RNAPII retained by the column also contained PP1.
This provides direct evidence that PP1 and RNAPII are part of the same macromolecular complex and confirms our earlier observations that PP1c is part of the preinitiation complex formed on HIV-1 promoter (9). PP1 remains associated with RNAPII during the initiation and early elongation step of HIV-1 transcription (9).

The regulatory subunit that targets PP1 to the RNAPII complex has not yet been identified. NIPPI is a possible candidate because it associates with PP1 with high affinity and is targeted to nuclear speckles that also contain RNAPII (12). This view is also supported by our earlier observation that NIPPI and PP1 were components of Tat-associated CTD kinase complex in T-lymphocytes (10). Taken together, our data imply that purified PP1 dephosphorylates both recombinant CTD domain of RNAPII. Our observations that PP1c is part of the preinitiation complex and confirms our earlier report is the first demonstration that the dephosphorylation of the CTD domain of RNAPII. Our gift of flavopiridol.

Dr. J. Brady (NCI, National Institutes of Health, Bethesda, MD) for the preparation of antibodies to PP1 and dephosphorylate it when kinases are blocked by EDTA or by flavopiridol.

To analyze whether protein phosphatases of the PP family contribute to the dephosphorylation of RNAPII in vivo, we used okadaic acid, a cell-permeable inhibitor of this family of protein phosphatases (5, 13). We found that a preincubation of the cells with 1 μM okadaic acid, but not 10 nM okadaic acid, partially prevented the dephosphorylation of RNAPII in intact cells. Since okadaic acid is a more potent inhibitor of PP2A than of PP1, these data are in agreement with a role for PP1 in the dephosphorylation of the CTD domain of RNAPII. Our report is the first demonstration that the dephosphorylation of RNAPII is mediated by PP1 in vitro and probably also in vivo. We also have preliminary data indicating that the overexpression of NIPPI blocks Tat-dependent HIV-1 transcription in transient transfection assays and that overexpression of PP1 alleviates this inhibitory effect of NIPPI. However, it remains to be seen whether the stimulatory role of PP1 on HIV-1 transcription also involves the dephosphorylation of the CTD domain of RNAPII or is explained by the stimulation of a post-transcriptional process.

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