TFIIF-associating Carboxyl-terminal Domain Phosphatase Dephosphorylates Phosphoserines 2 and 5 of RNA Polymerase II*

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The carboxyl-terminal domain (CTD) of the largest RNA polymerase (RNAP) II subunit undergoes reversible phosphorylation throughout the transcription cycle. The unphosphorylated form of RNAP II is referred to as IIA, whereas the hyperphosphorylated form is known as IIO. Phosphorylation occurs predominantly at serine 2 and serine 5 within the CTD heptapeptide repeat and has functional implications for RNAP II with respect to initiation, elongation, and transcription-coupled RNA processing. In an effort to determine the role of the major CTD phosphatase (FCP1) in regulating events in transcription that appear to be influenced by serine 2 and serine 5 phosphorylation, the specificity of FCP1 was examined. FCP1 is capable of dephosphorylating heterogeneous RNAP IIO populations of HeLa nuclear extracts. The extent of dephosphorylation at specific positions was assessed by immunoreactivity with monoclonal antibodies specific for phosphoserine 2 or phosphoserine 5. As an alternative method to assess FCP1 specificity, RNAP IIO isozymes were prepared in vitro by the phosphorylation of purified calf thymus RNAP IIA with specific CTD kinases and used as substrates for FCP1. FCP1 dephosphorylates serine 2 and serine 5 with comparable efficiency. Accordingly, the specificity of FCP1 is sufficiently broad to dephosphorylate RNAP IIO at any point in the transcription cycle irrespective of the site of serine phosphorylation within the consensus repeat.

Reversible phosphorylation of the carboxyl-terminal domain (CTD)§ of the largest RNA polymerase (RNAP) II subunit plays an important role in the regulation of gene expression. The CTD of mammalian RNAP II is comprised of 52 repeats of the consensus sequence YSPTSPS¶ (for a review, see Ref. 1). RNAP IIA, which contains an unmodified CTD, is actively recruited to the promoter as part of the preinitiation complex (2–5), whereas RNAP IIO, which contains a hyperphosphorylated CTD, is responsible for transcript elongation (6, 7). Therefore, protein kinases and phosphatases that alter the state of CTD phosphorylation can serve as transcriptional activators or repressors depending on the point in the transcription cycle at which they function.

The unphosphorylated form of RNAP II is referred to as IIa, whereas the hyperphosphorylated form is known as IIO. Phosphorylation occurs predominantly at serine 2 and serine 5 within the CTD heptapeptide repeat and has functional implications for RNAP II with respect to initiation, elongation, and transcription-coupled RNA processing. In an effort to determine the role of the major CTD phosphatase (FCP1) in regulating events in transcription that appear to be influenced by serine 2 and serine 5 phosphorylation, the specificity of FCP1 was examined. FCP1 is capable of dephosphorylating heterogeneous RNAP IIO populations of HeLa nuclear extracts. The extent of dephosphorylation at specific positions was assessed by immunoreactivity with monoclonal antibodies specific for phosphoserine 2 or phosphoserine 5. As an alternative method to assess FCP1 specificity, RNAP IIO isozymes were prepared in vitro by the phosphorylation of purified calf thymus RNAP IIA with specific CTD kinases and used as substrates for FCP1. FCP1 dephosphorylates serine 2 and serine 5 with comparable efficiency. Accordingly, the specificity of FCP1 is sufficiently broad to dephosphorylate RNAP IIO at any point in the transcription cycle irrespective of the site of serine phosphorylation within the consensus repeat.

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§ The abbreviations used are: CTD, carboxyl-terminal domain; CTDa and CTDb, unphosphorylated and hyperphosphorylated CTD, respectively; RNAP II, RNA polymerase II; FCP, TFIIF-associating CTD phosphatase; TFI1, general transcription factor for RNA polymerase II; RAP, RNA polymerase II-associating protein; GST, glutathione S-transferase; P-TEFb, positive transcription elongation factor; MAP, mitogen-activated protein; MAPK2/ERK2, MAP kinase 2; DTT, dithiothreitol.

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CTD phosphorylation is maintained by the opposing actions of CTD kinases and CTD phosphatase(s). For example, Ctk1 (a putative P-TEFb homolog in yeast) and FCP1 (TFIIIF-associating CTD phosphatase) appear to modulate the level of serine 2 phosphorylation in the RNA Pol II elongation complex (25). In addition, the level of phosphorylation of nontranscribing RNA Pol II in Xenopus laevis early embryos is maintained by MAP kinase Xp42 and FCP1 (26).

Unlike many CTD kinases that have been discovered and characterized, a single CTD phosphatase has been reported to date (for a review, see Ref. 27). Genetic studies have demonstrated that FCP1 is required for transcription in vivo, and its inactivation leads to a global defect in mRNA synthesis (28). The dephosphorylation of RNA Pol II is dependent on the interaction of FCP1 with a site on RNA Pol II that is outside of the CTD (29, 30). FCP1 activity is stimulated by RAP74, the larger of the two subunits of TFIIF (29). TFIIF abrogates the stimulatory activity of TFIIH but has no influence on FCP1 activity in the absence of TFIIH.

FCP1 dephosphorylates RNA Pol II generated by serine/threonine CTD kinases but is not sensitive to vandanate, a tyrosine phosphatase inhibitor (31). Furthermore, the sensitivity of RNA Pol II in an elongation complex to FCP1 is dependent on its position with respect to the transcriptional start site (32, 33). Although it has been established that FCP1 dephosphorylates phosphoserine 2 at the 3′ end of the gene (25) and can recycle RNA Pol II to RNA Pol II A (34), it is unclear if FCP1 can dephosphorylate phosphoseryl 5 during transcript elongation (15). To understand the involvement of FCP1 at discrete stages in the transcription cycle, it is necessary to determine its subcellular specificity. To examine FCP1 specificity, two independent experimental approaches were used in this study. First, FCP1 activity was assayed toward endogenous RNA Pol II subunits contained in HeLa nuclear extracts. Second, FCP1 activity was assessed using purified calf thymus RNA Pol II substrates prepared in vitro by the phosphorylation of RNA Pol II A with different CTD kinases.

**EXPERIMENTAL PROCEDURES**

**Materials**—x-[32P]ATP (600 Ci/mmol) was purchased from PerkinElmer Life Sciences. Human recombinant casein kinase II and mouse recombinant MAP kinase 2/ERK2 (MAPK2/ERK2) were obtained from Upstate Biotechnology, and human recombinant Cdc2 kinase was purchased from New England Biolabs. Human CTDK1/CTDK2 were partially purified as previously described (35). Human TFIIH was gener-
ariously provided by Dr. Jean-Marc Egly (36). Human P-TEFb was partially purified from HeLa S-100 extract by chromatography on heparin-Sepharose (Amersham Biosciences), DEAE 15HR (Millipore), Hi-
Trap S, and Phenyl-Superose (both from Amersham Biosciences). P-
TEFb was dialyzed against buffer A (25 mM Hepes, pH 7.9, 20% glycerol, 25 mM KCl, 0.1 mM EDTA, 1 mM DTT, 1 mM phenylmethyl-
sulfonyl fluoride). Monoclonal Antibodies—Monoclonal antibody POL3/3 recognizes a conserved epitope within the largest RNA Pol II subunit that is distinct from the CTD (37, 38). H5 and H14 are IgMs directed against phosphoseryl residues within the CTD (39–41) and were obtained from Covance. CC3 is an IgG isolated in a screen for chicken proteins with developmentally regulated expression (42). B3 is an IgM directed against nuclear matrix components (43).

**Preparation of HeLa Nuclear Extracts**—HeLa nuclear extracts were prepared from control cells and cells treated with actinomycin D at 1 μg/ml for 1 h or serum-deprived for 24 h and stimulated with 20% serum for 1 h. After their respective treatments, the cell monolayers grown in 150-cm² dishes were washed with cold phosphate-buffered saline and gently removed by scraping in buffer B (10 mM Hepes, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT). Cells were centrifuged at 1,000 × g for 10 min, and the pellets were resuspended in buffer B on ice. The cells were then homogenized with a Dounce homogenizer (10 times), and the lysates were centrifuged at 10,000 × g for 10 min and fractionated into nuclear pellets and cytosolic supernatants. The nuclear pellets were resuspended in buffer C (20 mM Hepes, pH 7.9, 1.5 mM MgCl₂, 25% glycerol, 420 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT) and centrifuged at 15,000 × g for 20 min. Pellets were re-suspended in buffer D (240 mM Hepes, pH 7.8, 1.5 mM MgCl₂, 25% glycerol, 1 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT) through a syringe to fragment the DNA and centrifuged at 15,000 × g for 20 min. The nuclear extracts were dialyzed against buffer E (50 mM Tris, pH 7.9, 20% glycerol, 120 mM KCl, 0.1 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride).

**Preparation and Purification of 32P-labeled RNA Pol II**—Calf thymus RNA Pol II A was purified by the method of Holo and Blatti (44) with modifications as described by Kang and Dahmus (5). Specific isozymes of 32P-labeled RNA Pol II A were prepared by phosphorylation of purified RNA Pol II A with recombinant casein kinase II and γ-[32P]ATP followed by incubation in the presence of excess unlabelled ATP (2 μM) with either purified CTDK1/CTDK2, TFIIH, P-TEFb, recombinant MAPK2/ERK2, or Cdc2 kinase. Each RNA Pol II isozyme was purified by step elution from DE53 as previously described (4). Because only the most carbonyl-terminal serine (casein kinase II site) is labeled with 32P and lies outside the consensus repeat, dephosphorylation by CTD phosphatase results in an electrophoretic mobility shift of subunit IIb to the position of subunit Iib without loss of label.

**CTD Kinase Assays**—CTD kinase assays were performed as described previously (35). Reactions were performed in 20 μl of CTD kinase buffer (20 mM Hepes, pH 7.8, 8 mM MgCl₂, 0.5% glycerol, 0.1% Triton X-100, 1 mM DTT). Each reaction contained 2.75 fmol of 32P-
labeled GST-CTD and an equivalent molar amount of 32P-labeled RNA Pol IIA. Reactions were initiated by the addition of either TFIIH, P-TEFb, recombinant MAPK/ERK2, or Cdc2 kinase. Each RNA Pol II isozyme was purified by gel filtration on a Superose 12 column and analyzed by autoradiography. The corresponding blot and gel images were scanned on a Molecular Dynamics Image Scanner Storm 860 in the phosphor screen mode and analyzed by ImageQuant software.

**CTD Phosphatase Assays**—Both purified and recombinant human FCP1 were used in these studies. Human FCP1 was purified from HeLa cells as described previously (45) and used in assays involving endogenous RNA Pol II isozymes. Recombinant human FCP1 was expressed in SF21 cells, purified to homogeneity, and used in assays involving RNA Pol II substrates prepared in vitro by CTD kinases. Recombinant FCP1 was purified by chromatography on Ni²⁺-nitrilotriacetic acid agarose (Qiagen) and HiTrap S-300 columns (Amersham Biosciences) and HiTrap H-250s/nuclease-activated column that had been coupled with recombinant RAP74 (Amersham Biosciences). Purified FCP1 had a specific activity of 40,000 units/mg, whereas recombinant FCP1 had a specific activity of 270,000 units/mg. One unit of CTD phosphatase corresponds to the activity required to convert 1 pmol of free RNA Pol II to RNA Pol II A in 1 min in the presence of a saturating amount of RAP74.

CTD phosphatase assays were performed as described previously (31) with minor modifications. Reactions were performed in 20 μl of CTD kinase buffer (50 mM Tris, pH 7.9, 10 mM MgCl₂, 20% glycerol, 0.025% Tween 80, 0.1 mM EDTA, 5 mM DTT) in the presence of 25 fmol of purified casein kinase II or HiTrap H-250s/nuclease-activated column that had been coupled with recombinant RAP74 (Amersham Biosciences). Reactions involving endogenous RNA Pol II in HeLa nuclear extracts contained 200–250 fmol of RNA Pol II. Reactions involving purified RNA Pol II contained 18 fmol of RNA Pol II. Both reactions were carried out in the presence of 7 pmol of RAP74. Reactions were initiated by the addition of FCP1 and incubated at 30 °C for 30 min. Assays were terminated by the addition of 5× Laemmli buffer, and RNA Pol II subunits were resolved on a 5% SDS-PAGE gel. CTD phosphatase assays of endogenous RNA Pol II isozymes contained in nuclear extracts were analyzed by Western blots using dilutions of 1:1,000 POL3/3, 1:250 HS, 1:250 H14, 1:1,000 CC3, or 1:1,000 B3 followed by 1:10,000 anti-mouse IgG horseradish peroxidase-conjugated secondary antibody (Promega). The blots were visualized by ECL Plus detection reagents (Amersham Biosciences). CTDPK phosphatase assay of purified casein kinase II-labeled RNA Pol II was analyzed by autoradiography. The corresponding blot and gel images were scanned on a Molecular Dynamics Image Scanner Storm 860 in blue fluorescence mode and phosphor screen mode, respectively.

**RESULTS**

FCP1 Dephosphorylates Phosphoserine 2 and Phosphoserine 5 within the CTD of Endogenous RNA Pol II—FCP1 specificity was initially examined with in vivo populations of heterogeneous RNA Pol II A contained in HeLa nuclear extracts. The reactivity of endogenous RNA Pol II with phosphoserine-specific monocalonal antibodies was assessed before and after dephosphorylation with FCP1. Based on reactivity with synthetic peptides, monocular antibodies H5 and CC3 recognize phosphoserine in position 2.
within the heptapeptide repeat, whereas H14 and B3 recognize phosphoserine in position 5 (Fig. 1A) (13). POL3/3, directed against an epitope in the largest RNAP II subunit that is outside of the CTD (37, 38), permits the detection of the largest subunit irrespective of the state of CTD phosphorylation. The degree and specificity by which FCP1 dephosphorylates endogenous RNAP II were measured by the disappearance of immunoreactivity. Because a variety of studies have shown that changes in growth conditions can give rise to changes in the level and pattern of CTD phosphorylation, HeLa nuclear extracts from different growth conditions can give rise to changes in the specificity by which FCP1 dephosphorylates endogenous RNAP II. As indicated by the disappearance of immunoreactivity of H5 and H14, FCP1 is capable of removing phosphates from serine 2 and serine 5 in control HeLa nuclear extract (Fig. 1B, panels H5 and H14, lanes 1 and 2). Likewise, FCP1 shows similar specificity toward RNAP IIA in HeLa nuclear extract from cells treated with actinomycin D (Fig. 1B, panels H5 and H14, lanes 3 and 4). Interestingly, phosphoserine 2 is relatively resistant to FCP1 dephosphorylation in HeLa nuclear extract from cells stimulated with serum (Fig. 1B, panels H5 and H14, lanes 5 and 6). These observations are confirmed by the use of CC3 and B3 in parallel Western blots (Fig. 1B, panels CC3 and B3). In the presence of higher concentrations of FCP1, the complete dephosphorylation of phosphoserine 2 in serum-stimulated HeLa nuclear extract is observed (data not shown). These results indicate that FCP1 is capable of removing phosphates from serine positions 2 and 5.

To investigate whether the resistance of phosphoserine 2 to FCP1 dephosphorylation is conferred with increasing time of serum exposure, HeLa cells were serum-starved for 24 h, and nuclear extracts were prepared from cells treated with 20% serum for 0, 10, 30, and 60 min. The percentage of RNAP IIO that remained relatively resistant to dephosphorylation did not change as a function of time, suggesting that resistance of phosphoserine 2 to FCP1 dephosphorylation is conferred by serum starvation rather than serum stimulation (data not shown).

TFIIFH and P-TEFb Preferentially Phosphorylate the CTD of RNAP IIA Relative to GST-CTDa—An alternative approach to establishing the specificity of FCP1 is to examine the ability of FCP1 to dephosphorylate RNAP IIO prepared in vitro by the phosphorylation of RNAP IIA with distinct CTD kinases. The phosphorylation of purified calf thymus RNAP IIA and GST-CTDa in the presence of increasing amounts of TFIIFH (lanes 1–5), P-TEFb (lanes 6–10), and MAPK2/ERK2 (lanes 11–15) is shown in Fig. 2. Both RNAP IIA and GST-CTDa are labeled with 32P at their terminal serine by phosphorylation with casein kinase II. Because RNAP IIA and GST-CTDa are present in equimolar amounts in the same reaction, the efficiency with which each is shifted to the phosphorylated form is a measure of the substrate specificity of the CTD kinase present. The relative difference in the intensity of radiolabeled GST-CTDa and radiolabeled subunit IIA is a consequence of the difference

![Image](https://example.com/image.png)
in the efficiency of $^{32}$P incorporation by casein kinase II.

TFIHI and P-TEFb efficiently convert RNAP IIA to RNAP IIO in a processive manner but show no or marginal activity toward GST-CTDAs, respectively (Fig. 2, lanes 1–5 and lanes 6–10). However, MAPK2/ERK2 converts both RNAP IIA and GST-CTDAs to their phosphorylated forms in a distributive manner and with comparable efficiency (Fig. 2, lanes 11–15). This result indicates that the activities of TFIHI and P-TEFb are strongly dependent on the context in which the CTD is presented. In contrast, the activity of MAPK2/ERK2 appears to be insensitive to context. These findings suggest that the activities of TFIHI and P-TEFb are dependent on factors that are extrinsic to the CTD. Accordingly, reaction parameters defined on the basis of synthetic peptides or non-native substrates may differ significantly from those determined utilizing native RNAP IIA as substrate.

**FCP1 Dephosphorylates RNAP IIO Prepared by Different CTD Kinases**—The ability of FCP1 to dephosphorylate isozymes of purified calf thymus RNAP IIO, prepared by the in vitro phosphorylation of RNAP IIA with CTDK1/CTDK2, TFIHI, P-TEFb, MAPK2/ERK2, and Cdc2 kinase, was examined (Fig. 3A). A quantitation of the dephosphorylation of RNAP IIO as a function of increasing amounts of FCP1 is shown in Fig. 3B. RNAP IIO prepared by each of the five CTD kinases is efficiently dephosphorylated by FCP1. Furthermore, a comparable amount of FCP1 is required to dephosphorylate RNAP IIO prepared by each of the CTD kinases examined. Finally, the absence of subunits with mobilities intermediate between that of subunits IIA and IIO indicates that FCP1 processively dephosphorylates each isozyme of RNAP IIO. Kinetic assay, carried out at a fixed FCP1 concentration (5 million units), confirmed that FCP1 dephosphorylates RNAP IIO prepared by different CTD kinases with comparable efficiency (data not shown).

**FCP1 Dephosphorylates RNAP IIO Prepared by TFIH or Cdc2 Kinase at pH 7.9 but Not at pH 5.5**—Synthetic peptides have been used extensively to characterize the activity of CTD kinases and more recently the activity of yeast FCP1 (50). Because of the reported low pH optimum of 5.5 required by yeast FCP1 to dephosphorylate synthetic peptides, the ability of human FCP1 to dephosphorylate isozymes of purified calf thymus RNAP IIO at pH 7.9 and pH 5.5 was examined (Fig. 4). FCP1 (50 million units) successfully converts RNAP IIO, prepared by the phosphorylation of RNAP II with either TFIHI or Cdc2 kinase, to RNAP IIA at pH 7.9 (lanes 1–4), whereas an equivalent amount of FCP1 shows no activity at pH 5.5 (lanes 5–8). This observation indicates that FCP1 activity toward native RNAP IIO is optimal near neutral or physiological pH.

**The Specificity of FCP1 Phosphatase**—The observation that the mobility of CTD kinases can differ in their relative activity with RNAP IIA and GST-CTDAs suggests that their specificity may be directly influenced by determinants in RNAP II that lie outside the CTD. Accordingly, it is important to know if the preferential phosphorylation of either serine 2 or 5 observed in synthetic peptides by distinct CTD kinases is also true when RNAP IIA serves as substrate. The ability of phosphoserine 2- or phosphoserine 5-specific monoclonal antibodies to react with RNAP IIO prepared by the phosphorylation of $^{32}$P-labeled RNAP IIA with TFIHI, P-TEFb, MAPK2/ERK2, Cdc2 kinase, and CTDK1/CTDK2 was examined. The upper panel of Fig. 5 ( Autoradiogram, lanes 2–6; see also Fig. 3A, lane 1) confirms that each CTD kinase efficiently converts RNAP IIA to IIO. There is a subtle variation in the mobility of the largest subunits among the five RNAP IIO isozymes prepared by the different CTD kinases. This variation suggests that the RNAP IIO isozymes may differ in phosphate stoichiometry, pattern of CTD phosphorylation, and/or structural conformations that can give rise to changes in gel mobility. The Western transfer of these same samples and reaction with POL3/3 shows a broader distribution of the largest subunit with mobilities intermediate between that of subunits IIA and IIO (Fig. 5, panel POL3/3). However, results presented in the upper panel of Fig. 5 show that casein kinase II-labeled RNAP IIA is quantitatively converted to fully phosphorylated RNAP IIO. This observation suggests that the fraction of RNAP IIA phosphorylated at the casein kinase I site is preferentially phosphorylated by each CTD kinase. Accordingly, the casein kinase II site may play a role in the regulation of RNAP II phosphorylation.

**DISCUSSION**

It has become increasingly clear that reversible phosphorylation of serine 2 and serine 5 in the CTD consensus repeat plays a critical role in the progression of RNAP II through the transcription cycle and in coupling RNA processing with transcript elongation. However, a precise definition of the changes in phosphorylation pattern that occur has been difficult due to the repetitive nature of the CTD. Synthetic peptides have played a key role in establishing the specificity of distinct CTD kinases as well as the specificity of monoclonal antibodies that have been used as structural probes. It is now clear that the preference of CTD kinases for serine phosphorylation at positions 2 or 5 can be influenced by factors extrinsic to the kinase. For instance, Ramanathan and co-workers (51) report that Cdk9 (enzymatic subunit of P-TEFb) preferentially phosphorylates serine 2 and a lower propensity to phosphorylate serine 5. The relative reactivity of Cdc2 kinase-phosphorylated RNAP IIO with phosphoserine 2-specific monoclonal antibodies (H5 and CC3) is 2–4 times higher than that of the other RNAP IIO isozymes. Conversely, Cdc2 kinase-phosphorylated RNAP IIO has a markedly reduced reactivity with phosphoserine 5 monoclonal antibodies (H14 and B3) relative to the other RNAP IIO isozymes. The results indicate that Cdc2 kinase has a higher propensity to phospho-rylate serine 2 and a lower propensity to phosphorylate serine 5 than do the other CTD kinases tested.
Because the specificity of CTD kinases is context-dependent and can be influenced by associating factors or even the substrate itself, experiments were carried out to determine their relative activity with respect to the phosphorylation of RNAP IIA and GST-CTDa. In the presence of equimolar amounts of RNAP IIA and GST-CTDa, both TFIIH and P-TEFb preferentially convert RNAP IIA to RNAP IIO while showing no activity or marginal activity toward GST-CTDa, respectively. Con-

**FIG. 3.** FCP1 dephosphorylation of RNAP IIO isoenzymes prepared by different CTD kinases. A, CTD phosphatase reactions of FCP1 were carried out as described under “Experimental Procedures.” Each reaction contained ~18 fmol of RNAP IIO prepared by a specific CTD kinase, 7 pmol of RAP74, and a specified amount of FCP1 (increasing from lane 1 to lane 6). B, quantitation of the percentage of subunit Ilo remaining as a function of FCP1 concentration. mU, milliunits.
substrate specificity of FCP1 phosphatase.

**Fig. 4.** FCP1 activity at pH 7.9 and pH 5.5. RNAP IIO, prepared by the phosphatase of 32P-labeled RNAP IIA with TFIIH or Cdc2 kinase, was incubated in the presence of increasing amounts of FCP1 at pH 7.9 or 5.5 and analyzed as described under “Experimental Procedures.” Each reaction contained ~18 fmol of RNAP IIO, 7 pmol of RAP74, and a specified amount of FCP1 (increasing from lane 1 to 4 and from lane 5 to 8) at either pH 7.9 (lanes 1–4) or pH 5.5 (lanes 5–8). mU, milliunits.

**Fig. 5.** Reaction of RNAP IIO isozymes with phosphoserine-specific monoclonal antibodies. Ten pmol of purified calf thymus 32P-labeled RNAP IIA (lane 1) was converted to RNAP IIO by CTDK1/CTDK2 (lane 2), TFIIH (lane 3), P-TEFb (lane 4), MAPK2/ERK2 (lane 5), and Cdc2 kinase (lane 6). The top panel is an autoradiogram showing the conversion of RNAP IIA to RNAP IIO by the various CTD kinases. The subsequent panels are the corresponding Western blots showing the reactivity with different monoclonal antibodies. Quantitation of the RNAP IIO immunoreactivity to phosphoserine 2- and phosphoserine 5-specific monoclonal antibodies is indicated as phosphor-stimulated luminescence per unit area above each lane.

versely, MAPK2/ERK2 phosphorylates RNAP IIA and GST-CTDα with nearly identical efficiency. Finally, although both TFIIH and P-TEFb convert RNAP IIA to RNAP IIO in a processive manner, MAPK2/ERK2 converts both RNAP IIA and GST-CTDα to their respective phosphorylated forms in a distributive manner.

To determine the substrate specificity of FCP1, two experimental approaches were used. The first approach takes advantage of the finding that changes in growth conditions result in changes in the pattern of CTD phosphorylation (12–14). This study demonstrates that FCP1 can dephosphorylate endogenous RNAP IIO populations from HeLa nuclear extracts prepared from differentially treated cells. As determined by diminished reactivity with phosphoserine-specific monoclonal antibodies, FCP1 catalyzes the removal of phosphates from both serine 2 and serine 5.

In the second approach, a panel of RNAP IIO isozymes were individually prepared in vitro by the phosphorylation of purified calf thymus RNAP IIA with TFIIH, P-TEFb, MAPK2/ERK2, Cdc2 kinase, and CTDK1/CTDK2. The pattern of phosphorylation by each CTD kinase was examined using a panel of monoclonal antibodies specific for serine 2 or serine 5 phosphorylation. The results presented here suggest that TFIIH, P-TEFb, and MAPK2/ERK2 have a higher propensity to phosphorylate serine 5 than does Cdc2 kinase. Conversely, Cdc2 kinase has a higher propensity to phosphorylate serine 2 than do the other CTD kinases. This is supported by the finding that the relative phosphoserine 2/phosphoserine 5 reactivity is similar among the panel of RNAP IIO prepared by TFIIH, P-TEFb, and MAPK2/ERK2 but differs markedly from that of RNAP IIO generated by Cdc2 kinase. The unanticipated reactivity of H5 with RNAP IIO isozymes prepared with TFIIH, P-TEFb, and MAPK2/ERK2 may result from either partial phosphorylation at serine 2 or cross-reactivity of H5 with serine 5 in the context of native RNAP II.

Results presented here using free RNAP II as substrate are in general agreement with studies using synthetic CTD peptides (51, 54, 55). TFIIH, P-TEFb, and MAPK2/ERK2 all phosphorylate free RNAP II at serine 5, whereas Cdc2 kinase phosphorylates the two substrates at both serines 2 and 5. As noted above, Zhou et al. (23) report that P-TEFb phosphorylates serine 2 in RNAP II assembled in a preinitiation complex. Just as the ability of FCP1 to dephosphorylate RNAP II differs dramatically between free RNAP II and RNAP II in an elongation complex (32, 33), the specificity of CTD kinases may differ between free RNAP II and RNAP II in specific protein-DNA complexes. Alternatively, RNAP II assembled in a preinitiation complex in the presence of HeLa nuclear extract may be associated with a factor(s) that alters the specificity of enzymes that modify the CTD.

Although RNAP IIO isozymes prepared by TFIIH and P-TEFb were slightly better substrates for FCP1 relative to RNAP IIO prepared by Cdc2 kinase, each RNAP IIO prepared in vitro can be efficiently dephosphorylated by FCP1 and successfully converted to RNAP IIA. This result indicates that FCP1 can remove phosphates from both serine positions 2 and 5. FCP1 specificity determined here using purified calf thymus RNAP IIO made by known CTD kinases corroborates the specificity established using endogenous RNAP IIO contained in HeLa nuclear extracts. These findings establish that FCP1 has a broad specificity and is capable of dephosphorylating different isozymes of RNAP IIO present at different stages in the transcription cycle.
A recent study on the specificity of Schizosaccharomyces pombe FCP1, on which phosphate release from synthetic CTD peptides was determined, report that FCP1 preferentially dephosphorylates phosphoserine 2 over phosphoserine 5 (50). The apparent discrepancy between these results and those presented here might be a consequence of differences in the source of FCP1, the substrate, and/or reaction conditions. In the study reported here, human FCP1 was assayed at pH 7.9 with native RNAP IIO substrates, whereas the study by Hausmann and Shuman (50) used fusion yeast FCP1 at pH 5.5 with phospho-CTD peptides. The pH optimum for yeast FCP1 hydrolysis of p-nitrophenol phosphate and phospho-CTD peptides is 5.5, whereas the pH optimum for human FCP1 toward native RNAP IIO is about 7.9, with no apparent activity at pH 5.5. Furthermore, in the presence of equimolar amounts of RNAP IIO and GST-CTDo (both prepared by MAPK2/ERK2), FCP1 successfully dephosphorylates native polymerase but not GST-CTDø at pH 7.9 (data not shown). These results indicate that at near neutral or physiological pH, free native RNAP II, but not the recombinant CTD, is the preferred substrate for FCP1. Last, the amount of FCP1 required to dephosphorylate RNAP IIO at pH 7.9 is substantially less than the amount used for the dephosphorylation of phospho-CTD peptides at pH 5.5.

The pattern of CTD phosphorylation can influence the recruitment of RNAP II to the promoter as well as coordinate transcript elongation and RNA processing. Using chromatin immunoprecipitation in yeast, Komarnitsky et al. (15) demonstrate that serine 5 phosphorylation is primarily detected at the promoter region, whereas serine 2 phosphorylation is observed in the coding region. Due to limitations on the use of monoclonal antibodies as structural probes, it is still unclear whether phosphates on serine 5 are removed before the addition of phosphates on serine 2. For example, it is possible that phosphorylation at position 2 alters the affinity of monoclonal antibodies directed against position 5 and vice versa.

The observation that serine 2 phosphorylation increases in promoter distal regions in fep1 mutants indicates that FCP1 is responsible for the turnover of serine 2 phosphates during transcript elongation (25). The results here showing that FCP1 can remove phosphates from serine 2 are in agreement with these studies and are consistent with the idea that FCP1 can dephosphorylate RNAP IIO concomitantly with or shortly after termination to replenish the pool of RNAP IIA (34). Conversely, the finding that serine 5 phosphorylation is not altered in fep1 mutants suggests that FCP1 may not participate in the dephosphorylation of phosphoserine 5 during early elongation (25). Using similar assays with some modifications, Schroeder et al. (22) found that FCP1 can modulate phosphoserine 5 levels and direct the dissociation of capping enzymes. Differences in the temperature at which the fep1 mutants were assayed may contribute to the discrepancy in these results.

It is important to definitively establish whether serine 5 phosphates turnover either partially or completely during early elongation. If serine 5 phosphates are indeed removed early during transcript elongation, the broad specificity of FCP1 makes it a likely candidate for acting at this stage of transcription. However, it is possible that another CTD phosphatase is responsible for the turnover of serine 5 phosphates and that FCP1 activity is down-regulated in early elongation. The broad specificity of FCP1 also makes it a likely candidate for 1) modulating RNAP II activity during different stages of transcription and 2) participating in the mobilization of RNAP IIO from storage sites. The key to understanding FCP1 involvement in the regulation of gene expression lies in determining the factors that influence its recruitment to different RNAP II-containing complexes.
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