

Identifying PhosphoCTD-Associating Proteins

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Summary

The C-terminal repeat domain (CTD) of the largest subunit of RNA polymerase II is hyperphosphorylated during transcription elongation. The phosphoCTD is known to bind to a subset of RNA processing factors and to several other nuclear proteins, thereby positioning them to efficiently carry out their elongation-linked functions. The authors propose that additional phosphoCTD-associating proteins (PCAPs) exist and describe a systematic biochemical approach for identifying such proteins. A binding probe is generated by using yeast CTD kinase I to exhaustively phosphorylate a CTD fusion protein. This phosphoCTD is used to probe fractionated yeast or mammalian extracts in a Far Western protein interaction assay. Putative PCAPs are further purified and identified by mass spectrometry.

Key Words

RNA polymerase II; CTD; CTD kinase I; PCAPs; phosphoCTD; transcription elongation; RNA processing; nuclear organization; hyperphosphorylation; Far Western; protein interaction blot; protein–protein interaction.

1. Introduction

The C-terminal repeat domain (CTD) of elongating RNA polymerase II (RNAP II) is highly phosphorylated, principally on Ser2 and Ser5 of the consensus repeats YSPTSPS. It is thought that the kinase activity of TFIIF adds phosphates onto Ser5 positions in conjunction with the initiation process and that subsequently an “elongation” CTD kinase (yeast CTDK-I or mammalian P-TEFb) subsequently adds multiple phosphates onto Ser2 positions in association with the commitment to effective elongation (**1**). It has been found that phosphorylation of the CTD leads to the binding of factors involved in RNA processing and in other nuclear events (**2–4**). These findings suggest that one significant role of the phosphoCTD is to spatially and functionally organize nuclear components associated with transcription. The phosphoCTD is well

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sued to this role, as it probably exists in a largely extended state; also in yeast and mammals it potentially extends from the polymerase more than 600 Å and 1200 Å, respectively (4–6).

The first few phosphoCTD-associating proteins (PCAPs) identified were uncovered using a yeast two-hybrid approach (7). After the CTD was shown to be required for pre-mRNA processing, the next several PCAPs were discovered among known pre-mRNA processing factors (8–11). After these discoveries, the authors felt that only a fraction of the PCAPs actually present in nuclei had been discovered and that the surface of the PCAP-ome had barely been scratched. Proposing that many more PCAPs remained to be found, the authors developed a biochemical approach to identify proteins likely to be associated with elongating RNAP II. The authors used CTDK-I (12–14), the yeast CTD kinase known to be involved in phosphorylating elongating polymerase (15), to generate a fully phosphorylated recombinant CTD fusion protein that was then employed as a binding probe to identify PCAPs. Using this approach, the authors have identified a number of new PCAPs in both yeast and mammalian cells, most of which appear to be involved with elongation-coupled events (4,6,16–18). However, the authors predict that still more elongation-related PCAPs remain to be found and propose that finding and characterizing them will lead to new insights into nuclear functional organization. It is also of note that there are three other known CTD kinases in yeast, with functions and specificities different from those of CTDK-I (1). It is expected that use of the other kinases to prepare a phosphoCTD probe will lead to the discovery of distinct sets of PCAPs.

Here, the authors' approach for identifying phosphoCTD-associating proteins is presented.

2. Materials

2.1. Preparation of Mammalian Extracts

1. Wash buffer: 20 mM HEPES-KOH, pH 7.5, 5 mM KCl, 0.5 mM MgCl₂, 0.2 M sucrose. The following are added just before use: 0.5 mM DTT, 0.1% PMSF (1:1000 dilution of saturated solution in isopropanol), protease inhibitors appropriate to the starting material.
2. Hypotonic buffer: Follow instructions for wash buffer, but omit sucrose.
3. Extraction buffer: 50 mM HEPES-KOH, pH 7.5, 10% sucrose, plus the following added just before use: 0.5 mM DTT, 0.1% PMSF (1:1000 dilution of saturated solution in isopropanol), protease inhibitors appropriate to the starting material.
4. HGE(0.5): 25 mM HEPES, pH 7.6, 15% glycerol, 0.1 mM EDTA, 0.5 M NaCl. The following are added just before use: 0.5 mM DTT, 0.1% PMSF (1:1000 dilution of saturated solution in isopropanol), protease inhibitors appropriate to the starting material.

2.2. Preparation of Yeast Extracts

1. Commercial yeast blocks: Eagle brand, moist cakes, used for baking.
2. Extract buffer: 25 mM Tris-HCl, pH 7.6, 25 mM KCl, 1 mM EDTA. The following are added just before use: 1 mM DTT, 0.1% PMSF (1:1000 dilution of saturated solution in isopropanol), protease inhibitors appropriate to the starting material.
3. Polyethylenimine (Polymix P): 10% (v/v) solution adjusted to pH 7.9.
4. HGE: 25 mM HEPES, pH 7.6, 15% glycerol, 0.1 mM EDTA. The following are added just before use: 1 mM DTT, 0.1% PMSF (1:1000 dilution of saturated solution in isopropanol), protease inhibitors appropriate to the starting material.

2.3. Detecting PCAPs by Far Western Blotting

1. Electroblooming buffer: Tris/Glycine, 20% methanol (19).
2. BRB, blocking/renaturation buffer: PBS containing 3% nonfat dry milk, 0.2% Tween 20, 0.1% PMSF, 5 mM NaF.
3. PBS-Tw: PBS (10 mM Na-PO₄, pH 7.2, 150 mM NaCl) containing 0.2% Tween 20.
4. HEPES dialysis buffer: 10 mM HEPES-KOH, pH 7.6, 50 mM NaCl, 0.1 mM PMSF.

2.4. Preparation of Hyperphosphorylated CTD

1. 10X Reaction buffer: 250 mM HEPES-KOH, pH 7.6, 100 mM MgCl₂, 50 mM NaF.
2. 2% (v/v) Tween 20: store at 4°C, but check carefully for contamination before use.
3. γ -[³²P]ATP, 10 mCi/mL, specific activity 4500 Ci/mmol.
4. 3 mM ATP.
5. Quick Spin Protein Column (Roche Molecular Biochemicals).

2.5. Purifying CTDK-I

1. HGE(X): HGE containing X M KCl.
2. P11 phosphocellulose.
3. DE52 DEAE-cellulose.
4. MonoS column (FPLC, Amersham, Piscataway, NJ).

3. Methods

The ideal starting material contains a high percentage of the PCAPs present in the cells being studied. Thus, one goal of initial extract preparation is to retain PCAPs while discarding other constituents. The method described here for preparing mammalian extracts appears to meet this goal well; the initial steps enrich for elongationally engaged RNAP II0 under conditions that should not disrupt most PCAP-phosphoCTD associations. In contrast, the yeast extract used to date in the authors' laboratory was actually prepared for the purpose of purifying CTDK-I, and the early steps used in its preparation may have resulted in loss of some PCAPs (especially polymin-P precipitation). The authors are in the process of developing an alternative method for preparation of yeast extracts.

3.1. Preparation of Mammalian Cell Extracts

1. Grow cells by standard techniques appropriate for the cell line to the desired density (for HeLa, $4\text{--}7 \times 10^5/\text{mL}$), then harvest by centrifugation (8 min at 3000g for HeLa cells).
2. Resuspend cells in wash buffer (20 mL/L medium), distribute into centrifuge bottles, and pellet at 3300g for 5 min.
3. Resuspend the cells and swell in hypotonic buffer on ice for 10 min (3 mL per gram of cell pellet weight [20]).
4. Homogenize on ice using a Dounce homogenizer with a B pestle to 80–100% lysis and pellet nuclei at 4°C (2000g) for 5 min.
5. Decant the supernatant and resuspend the nuclear pellet in cold extraction buffer (1.5 mL per gram of cell pellet weight). Measure the volume of the resuspension and add 0.031 vol 5 M NaCl stock solution (final NaCl concentration approx 150 mM).
6. Rock or rotate the mixture at 4° for 1 h, then centrifuge at 4°C (14,500g) for 20 min.
7. Decant the supernatant (low-salt nuclear extract) and save if desired. Estimate the volume of the pellet (low-salt extracted nuclei) and add 8 vol of buffer HGE(0.5). Gently but thoroughly homogenize the pellet on ice (Dounce), then centrifuge the homogenate at 150,000g for 60 min at 4°C.
8. Decant and save the supernatant (0.5 M NaCl extract); it serves as the source of PCAPs. Residual pellet can be extracted with higher salt if desired (*see Fig. 1 in [4]*).

3.2. Preparation of Yeast Cell Extracts

Commercial baker's yeast is a convenient and inexpensive starting material if a specific genetically defined strain is not initially required. Baker's yeast moist cakes are crumbled into liquid nitrogen and stored in convenient aliquots at -80°C . Similarly, frozen batches of defined strains can also be used. The following methods are described in refs. **12** and **14**. All steps are carried out at 4°C.

1. Place approx 300 g of crumbled, frozen yeast in a stainless steel Waring blender (approx 3.8-L capacity) and add liquid nitrogen to cover yeast. Pulse on medium power in 30-s bursts. Monitor breakage by measuring soluble protein (Bradford assay, e.g., Bio-Rad), and continue pulverizing until soluble protein begins to plateau (usually 4–5 min).
2. After liquid nitrogen evaporates, scrape the yeast powder into a beaker and suspend it in 1 L extract buffer.
3. Centrifuge at 15,000g for 30 min and retain the supernatant.
4. With stirring, add a solution of 10% polyethylenimine, pH 7.9 to a final concentration of 0.3% to the supernatant, stir for an additional 30 min.
5. Centrifuge at 10,000g for 15 min and retain the pellet.
6. Resuspend the pellet in 200 mL extract buffer using Dounce homogenization and centrifuge again. Retain the pellet.

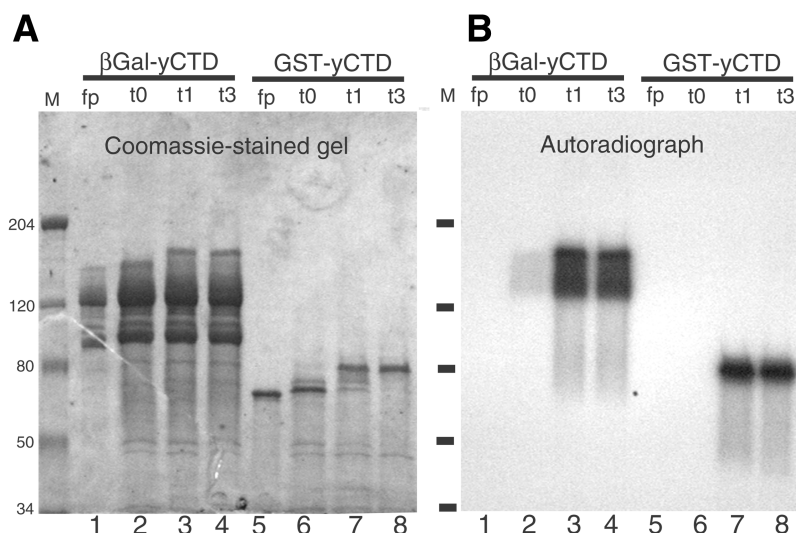


Fig. 1. Phosphorylation of yeast CTD fusion proteins by yeast CTDK-I. CTD fusion proteins (GST-yCTD and, separately, β Gal-yCTD) were incubated with CTDK-I as described in the text. Time points were taken at 0, 1, and 3 h (aliquots representing about 1/15 of the reaction = t0, t1, t3), heated in SDS sample buffer, and subjected to SDS-PAGE. (A) Coomassie blue-stained gel with marker proteins in lane *M* (kDa on left). Lanes 1 and 5 contain untreated fusion proteins. Note that intact β Gal-yCTD fusion protein is the top band in lanes 1–4, whereas faster-migrating bands between the intact form and approx 120 kDa are proteolyzed forms. (B) Autoradiograph of gel in (A). The t0 time points were taken a few seconds after adding unlabeled ATP to start the reaction. For the β Gal-yCTD reaction, radiolabeled ATP was added just before unlabeled ATP, whereas for the GST-yCTD reaction, unlabeled ATP was added first.

7. Extract kinase activity by homogenizing the pellet in 200 mL 0.4 *M* KCl in extract buffer using a Dounce homogenizer. Centrifuge homogenate at 10,000*g* for 15 min and retain the supernatant.
8. Stirring slowly, add solid ammonium sulfate to the supernatant to 45% saturation, stir 30 min, and centrifuge at 15,000*g* for 30 min. Resuspend the pellet in HE; dialyze against HGE for the minimum amount of time needed for the conductivity to reach that of HGE containing 0.15 *M* KCl. Centrifuge the dialysate at 15,000*g* for 10 min and retain the supernatant.
9. Divide the extract into aliquots and store at -80°C .

The authors are in the process of developing alternative methods for preparation of yeast extracts. One approach that has succeeded demonstrably in identifying proteins associated with elongating RNAP II (21–23) is that of Wootner and colleagues (24). This type of extract should also serve as a starting material for CTDK-I purification.

3.3. Detecting PCAPs by Far Western Blotting

1. Heat aliquots of the protein mixtures to be probed (e.g., 0.5 M NaCl extract or a column fraction) in SDS sample buffer according to standard procedures (e.g., 90°C for 5 min).
2. Subject the protein samples to SDS-PAGE (e.g., 4–15% gradient gels, Bio-Rad) along with prestained marker proteins (Bio-Rad).
3. Electroblot the proteins from the gel to supported nitrocellulose (Hybond-C Extra, Amersham Biosciences). Typically, the authors transfer for 2 h at 0.75 amp at 4°C using a Hoeffer TE50X apparatus.
4. Incubate the nitrocellulose overnight in BRB (above) plus 2 mM dithiothreitol (this and subsequent steps at 4°C).
5. Probe nitrocellulose with hyperphosphorylated CTD.
 - a. For [³²P]-labeled phosphoCTD, use at least 2.5 µg GST-phosphoCTD fusion protein (≥300,000 cpm; *see Note 4*) in 5 mL fresh BRB. Rock for 1–4 h at 4°C. Rinse the nitrocellulose membrane in PBS-Tw, 4 × 8 min, ≥50 mL per rinse, and monitor the washes until no radioactivity is detected. Blot dry by placing nitrocellulose on thick filter paper for a few seconds, protein side up. Cover with plastic wrap and expose to film or to a storage phosphor screen.
 - b. For nonradioactive phosphoCTD, use at least 2.5 µg probe (GST-phosphoCTD) in 5 mL fresh BRB. Rock and rinse as **step 5a**, above. Incubate with the primary antibody (e.g., rabbit anti-GST IgG) in fresh BRB for 30 min. Wash 3 × 10 min in PBS-Tw and incubate with the secondary antibody (e.g., peroxidase-coupled donkey anti-rabbit IgG) in fresh BRB for 30 min. Wash 4 × 10 min in PBS-Tw and develop. Antibody dilutions should be determined in trial experiments.

3.4. Preparation of the Hyperphosphorylated CTD Probe

3.4.1. Preparing [³²P]-Labeled PhosphoCTD

1. Express GST-CTD fusion protein in bacteria and purify using an affinity matrix (**25**); check the purity and amount of intact protein using SDS-PAGE (*see Note 1*). Dialyze the fusion protein into HEPES dialysis buffer. Store frozen in aliquots at –80°C.
2. Set up the kinase reaction by adding the following to the microfuge tube:
 - 10 µL 10X reaction buffer
 - x µL GST-CTD fusion protein (approx 25 µg)
 - 5 µL 2% Tween-20 solution
 - y µL CTD kinase (approx 200 ng enzyme)Add deionized H₂O to make 100 µL [72.5 – (x + y) µL]; this can also be added first.
 - 10 µL (100 µCi) g-[³²P]ATP
 - 2.5 µL 3mM ATP (final concentration = 75 µM).
3. Mix gently by micropipetting.
4. Immediately remove 2–5 µL, pipet into SDS sample buffer, and heat for 5 min at 90°C to generate the time zero point.

5. Incubate the kinase reaction at 30°C for 1 h.
6. Add 7.5 μL 3 mM stock nonradioactive ATP (final = 300 μM) (*see Note 3*).
7. Incubate at 30°C for an additional 60 min.
8. Remove 2–5 μL and pipet into SDS sample buffer; heat for 5 min at 90°C to generate the 2-h time point. Place the remainder of the kinase reaction at –80°C.
9. Check the extent of CTD phosphorylation by analyzing the $t = 0$ and $t = 2$ h samples, along with untreated fusion protein and prestained marker proteins, using SDS-PAGE (e.g., 4–12% or 4–15% acrylamide gradient gel). Electrophorese the samples until prestained standards of approximately the same size as the CTD fusion protein migrate two-thirds of the distance to the bottom of gel. Stain with Coomassie blue. Destain and monitor the mobility shift of fusion protein in the $t = 2$ h sample as compared to the untreated and $t = 0$ samples. *See Fig. 1* for examples of the time course of phosphorylation.
10. If all of the (intact) fusion protein is not shifted to slower mobility, it will be necessary to further kinase the sample. Thaw the remainder of the kinase reaction by gently agitating the tube in room temperature water until just thawed. Add another aliquot of CTD kinase (e.g., y μL or 0.5 y μL). Incubate at 30°C for an additional 1–2 h. Remove 2–5 μL and analyze by SDS-PAGE as before. To determine the extent of radioactivity that has been incorporated, the gels can be analyzed by autoradiography or PhosphorImager analysis (*see Fig. 1* for an example).
11. Following the kinase reaction, [^{32}P]-ATP is removed and the buffer is exchanged into PBS or other desired buffer using a 1-mL quick-spin protein column following the manufacturer's instructions. This procedure yields a final volume of about 400 μL . The authors assume a spin column yield of approx 80%. The phosphorylated CTD fusion protein is stored at –80°C. This procedure should yield sufficient probe for 4–8 Far Western blots.

3.4.2. Preparation of Nonradioactive PhosphoCTD

To prepare phosphoCTD without a radioactive label, proceed as above, but increase the initial concentration of nonradioactive ATP to 300 μM and omit radioactive ATP.

3.5. Purifying CTD Kinase

CTD kinases differ in the positions on the CTD to which they add phosphate groups, although a rigorous determination of this site specificity has yet to be carried out for any CTD kinase (*1*). Likewise, PCAPs display binding preferences for CTDs carrying phosphates in particular patterns. Thus, the set of PCAPs detected will depend on the CTD kinase used to prepare the phosphorylated CTD. The authors have used yeast CTDK-I exclusively in studies and expect that use of the yeast Kin28, Bur1, and Srb10 CTD kinases will result in the identification of different sets of proteins. It will also be interesting to note whether the use of a mammalian homolog of CTDK-I (e.g., P-TEFb) might result in the discovery of additional PCAPs.

3.5.1. Purification of CTDK-I From Yeast (**refs. 12,14**; see also **Subheading 3.2.**)

1. Apply extract (**Subheading 3.2.**) to phosphocellulose (P11) column (50-mL bed volume) equilibrated in HGE(0.15).
2. Wash the column extensively with the same buffer, then elute with a 250-mL linear gradient of 0.2–0.8 *M* KCl in HGE.
3. Assay fractions for CTDK-I activity (*see Subheading 3.5.2.*) and pool peak fractions (enzyme usually elutes at approx 0.32 *M* KCl).
4. Dialyze sample (vs HGE) to 0.12 *M* KCl and apply to a DE52 column (8-mL bed volume) equilibrated in the same buffer. The kinase is recovered in the flow-through fraction.
5. Apply the flow-through fraction directly to a 1-mL MonoS column and elute with a 0.15–0.45 *M* KCl gradient. CTDK-I elutes at approx 0.35 *M* KCl. Assay fractions for CTDK-I activity and pool fractions containing the peak of enzymatic activity.

3.5.2. Assay for CTD Kinase I (CTDK-I) Activity

1. For a 20- μ L reaction, add 2 μ L 10X reaction buffer (**Subheading 2.4.**), yeast CTD fusion protein to a final concentration of 0.15 mg/mL intact protein, 2–10 μ L of each column fraction, ATP to 300 μ M, γ -[32 P]ATP (1–3 μ Ci), and water to 20 μ L.
2. Incubate the mixture for 15–60 min at 30°C.
3. Terminate the reaction by adding SDS sample buffer and heating at 90°C for 5 min.
4. Analyze the products by SDS-PAGE followed by autoradiography (*see Fig. 1*).

3.6. Fractionation and Purification of PCAPs

The overall approach is four-pronged: (1) fractionate the extract, (2) test resulting fractions for presence of PCAPs, (3) fractionate/purify PCAPs further if necessary, and (4) identify the PCAPs.

1. Extracts are fractionated by conventional ion-exchange chromatography. A high-capacity cation-exchange resin that will handle large amounts of material and to which most PCAPs are expected to bind is a good choice. The authors have successfully used Macro-Prep High S, Macro-Prep CM (Bio-Rad, Hercules, CA), and HiTrap S (Amersham, Piscataway, NJ) supports (**4,18**). After application of the sample at low salt (e.g., 0.15 *M* KCl), the column is developed with a gradient of increasing salt concentration (e.g., 0.15–1.0 *M* KCl).
2. Identification of PCAPs in column fractions: An aliquot of each fraction is applied to duplicate SDS-PAGE gels. One gel is stained with Coomassie blue; the other is analyzed by Far Western blotting using a PCTD probe (*see above*). If a PCAP corresponds to a single protein band, it is excised from the stained gel and identified by mass spectrometry (*see Subheading 3.7.*). Fractions containing less pure PCAPs are fractionated further as described below.
3. Additional purification of PCAPs: PCAP-containing fractions are pooled, diluted to 0.15 *M* KCl, and applied to a second column. Good candidates for this column

are P-Serine-agarose (Sigma-Aldrich, St. Louis, MO) (**4**), or phosphoCTD-agarose (**18**). The second column is eluted with steps of increasing solvent concentration (e.g., phosphoserine, salt), and recovered fractions are analyzed on parallel SDS-PAGE gels as previously mentioned. Many PCAPs should now correspond to single protein bands and can be identified by mass spectrometry. Others may require one more fractionation step (e.g., MacroPrep High Q [Bio-Rad], [**4**]).

3.7. Identifying PCAPs by Mass Spectrometry

PCAPs are identified by excising the band from the Coomassie blue-stained gel corresponding to PCTD-binding activity (Far Western blot) and subjecting it to mass spectrometry analysis. The authors have submitted numerous samples to the Laboratory for Protein Microsequencing and Proteomic Mass Spectrometry, University of Massachusetts Medical School, Worcester Foundation Campus, Shrewsbury, MA (<http://www.umassmed.edu/proteomic/>) for in-gel trypsinization and MALDI mass spectrometry analysis. Note that mass spectrometry technology is rapidly evolving; check with experts for the current best approach.

3.8. Confirming PCAP Identity

The mass spectrometry-based identification of a PCAP needs to be checked by testing *bona fide* protein for its ability to bind the phosphoCTD. The most convincing approach is to demonstrate that purified, recombinant putative PCAP binds the phosphoCTD. If recombinant protein is not available conventionally-purified putative PCAP can be used.

3.8.1. Far Western Analysis With phosphoCTD as Probe

1. Obtain (express and purify) recombinant protein to be tested.
2. Separate the test protein on three individual SDS PAGE gels and blot all three gels onto nitrocellulose for Far Western analysis. Include positive (known PCAP) and negative (known non-PCAP) control proteins in the SDS gels.
3. One blot is probed with a phosphoCTD fusion protein (e.g., GST-phosphoCTD) as described above. A second blot is probed with the fusion partner (e.g., GST). Binding of the test protein to phosphoCTD fusion protein but not to the fusion partner confirms that the test protein identification was correct and that it is actually a PCAP. The third blot is probed with the nonphosphorylated CTD fusion protein (e.g., GST-CTD). The binding of the putative PCAP to the phosphoCTD fusion protein but not to the nonphosphorylated CTD fusion protein indicates that binding depends on CTD phosphorylation.

3.8.2. Far Western Analysis of PhosphoCTD Binding Using the Putative PCAP as Probe (Reverse Far Western, see **Note 5**)

1. To adjacent lanes of an SDS-PAGE gel, apply phosphoCTD fusion protein (e.g., GST-phosphoCTD), nonphosphorylated CTD fusion protein, and the fusion part-

- ner (e.g., GST) together with prestained marker proteins (*see Note 6*). Multiple sets should be applied to a single gel.
2. Electrophoresis and blot onto nitrocellulose membrane as described previously.
 3. Cut the nitrocellulose membrane to separate each set of three lanes.
 4. Probe one set of lanes with the recombinant putative PCAP (e.g., MBP-PCAP). A *bona fide* PCAP will bind to the phosphoCTD fusion protein (i.e., show a signal in lane 1) but not to the non-phosphoCTD or the fusion partner (i.e., no signal in lanes 2 and 3). For an example, *see* Phatnani and Greenleaf, 2004 (**18**).

4. Notes

1. A CTD fusion protein with affinity tags at both ends (e.g., GST-yCTD-His₆) affords the opportunity to use sequential affinity purifications to generate only full-length protein (**25**). However, a single affinity purification step frequently yields a preparation with sufficient full-length protein for many uses. Of course, the CTD can be fused to purification tags other than GST (*see Fig. 1*). Interestingly, in the authors' experience, the efficiency of phosphorylation of the CTD varies depending on its fusion partner; this phenomenon has not been thoroughly investigated.
The authors have observed that the nonfused CTD (i.e., free CTD, not attached to a protein fusion partner) displays some unusual properties. It is not stained well by either Coomassie blue or silver, and it is not easily detected after SDS-PAGE and Western blotting. On the other hand, because each CTD repeat contains a tyrosine, its absorbance at 280 nm can be easily measured.
2. If fully shifted, exhaustively phosphorylated fusion protein is required, small-scale trial experiments should be first performed to determine how much CTD kinase to use. This practice will help conserve kinase.
3. CTDK-I has an apparent K_m for ATP of approx 30 μM (**12**); incubation with total ATP concentration much below this level (e.g., using carrier-free radiolabeled ATP) results in very little incorporation. The authors attempt to maximize the specific radioactivity of the final product by incubating the kinase reaction for an hour with [ATP] somewhat above the K_m , and then for an additional 1 h or more at "saturating" [ATP].
4. To estimate the amount of [³²P]phosphoCTD-fusion protein, place 2 μL of the material recovered from the spin column in **Subheading 3.4.5.** at the bottom of a 1.5-mL microfuge tube. Place this against the detector of a Geiger counter and determine the counts per second. This figure is then converted to cpm/ μL . This method gives estimated cpm that are about six- to eightfold lower than counts determined by scintillation counter (using scintillation fluid).
5. In the authors' experience, segments of certain PCAPs subjected to SDS-PAGE and electroblotting to nitrocellulose sometimes fail to bind phosphoCTD fusion protein probe, presumably because the phosphoCTD-interacting domain does not renature under the conditions used. In such cases, a reverse Far Western approach can be used. The advantage is that the putative PCAP is used in its native (not denatured and renatured) state to probe the phosphoCTD fusion protein that has

been subjected to SDS-PAGE and electroblotting to nitrocellulose. The phosphoCTD is thought to be normally largely unstructured in solution (5) and therefore does not suffer irreversible denaturation during this procedure.

6. Exhaustively phosphorylated (i.e., fully shifted) CTD fusion protein is not absolutely necessary for the reverse Far Western approach. To conserve kinase, a small-scale phosphorylation reaction can be carried out on 1–2 μ g fusion protein for 1 h. If some nonshifted (hypophosphorylated) fusion protein remains, it can serve as an internal negative control for the dependence of binding on phosphorylation (e.g., lane 7 in **Fig. 1**).

References

1. Prelich, G. (2002) RNA polymerase II carboxy-terminal domain kinases: emerging clues to their function. *Eukaryotic Cell* **1**, 153–162.
2. Bentley, D. (2002) The mRNA assembly line: Transcription and processing machines in the same factory. *Curr. Opin. Cell. Biol.* **14**, 336–342.
3. Maniatis, T. and Reed, R. (2002) An extensive network of coupling among gene expression machines. *Nature* **416**, 499–506.
4. Carty, S. M. and Greenleaf, A. L. (2002) Hyperphosphorylated C-terminal repeat domain-associating proteins in the nuclear proteome link transcription to DNA/chromatin modification and RNA processing. *Mol. Cell. Proteomics* **1**, 598–610.
5. Cagas, P. M. and Corden, J. L. (1995) Structural studies of a synthetic peptide derived from the carboxyl-terminal domain of RNA polymerase II. *Proteins* **21**, 149–160.
6. Morris, D. P. and Greenleaf, A. L. (2000) The splicing factor, Prp40, binds the phosphorylated carboxyl-terminal domain of RNA polymerase II. *J. Biol. Chem.* **275**, 39,935–39,943.
7. Yuryev, A., Patturajan, M., Litington, Y., et al. (1996) The C-terminal domain of the largest subunit of RNA polymerase II interacts with a novel set of serine/arginine-rich proteins. *Proc. Natl. Acad. Sci. USA* **93**, 6975–6980.
8. McCracken, S., Fong, N., Yankulov, K., et al. (1997) The C-terminal domain of RNA polymerase II couples mRNA processing to transcription. *Nature* **385**, 357–361.
9. McCracken, S., Fong, N., Rosonina, E., et al. (1997) 5'-capping enzymes are targeted to pre-mRNA by binding to the phosphorylated carboxy-terminal domain of RNA polymerase II. *Genes Dev.* **11**, 3306–3318.
10. Cho, E. J., Takagi, T., Moore, C. R., and Buratowski, S. (1997) mRNA capping enzyme is recruited to the transcription complex by phosphorylation of the RNA polymerase II carboxy-terminal domain. *Genes Dev.* **11**, 3319–3326.
11. Yue, Z., Maldonado, E., Pillutla, R., Cho, H., Reinberg, D., and Shatkin, A. J. (1997) Mammalian capping enzyme complements mutant *Saccharomyces cerevisiae* lacking mRNA guanylyltransferase and selectively binds the elongating form of RNA polymerase II. *Proc. Natl. Acad. Sci. USA* **94**, 12,898–12,903.
12. Lee, J. M. and Greenleaf, A. L. (1989) A protein kinase that phosphorylates the C-terminal repeat domain of the largest subunit of RNA polymerase II. *Proc. Natl. Acad. Sci. USA* **86**, 3624–3628.

13. Lee, J. M. and Greenleaf, A. L. (1991) CTD kinase large subunit is encoded by *CTK1*, a gene required for normal growth of *Saccharomyces cerevisiae*. *Gene Expr.* **1**, 149–167.
14. Sterner, D., Lee, J. M., Hardin, S. E., and Greenleaf, A. L. (1995) Yeast carboxyl-terminal repeat domain kinase CTDK-I is a divergent cyclin–cyclin-dependent kinase complex. *Mol. Cell. Biol.* **15**, 5716–5724.
15. Cho, E. J., Kobor, M. S., Kim, M., Greenblatt, J., and Buratowski, S. (2001) Opposing effects of Ctk1 kinase and Fcp1 phosphatase at Ser 2 of the RNA polymerase II C-terminal domain. *Genes Dev.* **15**, 3319–3329.
16. Morris, D. P., Phatnani, H., and Greenleaf, A. L. (1999) Phospho-CTD binding and the role of a prolyl isomerase in pre-mRNA 3' end formation. *J. Biol. Chem.* **274**, 31,583–31,587.
17. Carty, S. M., Goldstrohm, A., Suñe, C., Garcia-Blanco, M. A., and Greenleaf, A. L. (2000) Protein-interaction modules that organize nuclear function: FF domains of CA150 bind the phospho-CTD of RNA polymerase II. *Proc. Natl. Acad. Sci. USA* **97**, 9015–9020.
18. Phatnani, H. P. and Greenleaf, A. L. (submitted) Novel phosphoCTD-associating proteins implicate CTD kinase I in diverse cellular processes.
19. Towbin, H., Staehelin, T., and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**, 4350–4354.
20. Longley, M. J., Pierce, A. J., and Modrich, P. (1997) DNA polymerase delta is required for human mismatch repair in vitro. *J. Biol. Chem.* **272**, 10,917–10,921.
21. Shi, X., Finkelstein, A., Wolf, A. J., Wade, P. A., Burton, Z. F. and Jaehning, J. A. (1996) Paf1p, an RNA polymerase II-associated factor in *Saccharomyces cerevisiae*, may have both positive and negative roles in transcription. *Mol. Cell. Biol.* **16**, 669–676.
22. Wade, P. A., Werel, W., Fentzke, R. C., et al. (1996) A novel collection of accessory factors associated with yeast RNA polymerase II. *Protein Expr. Purif.* **8**, 85–90.
23. Pokholok, D. K., Hannett, N. M., and Young, R. A. (2002) Exchange of RNA polymerase II initiation and elongation factors during gene expression in vivo. *Mol. Cell* **9**, 799–809.
24. Wootner, M., Wade, P. A., Bonner, J. and Jaehning, J. A. (1991) Transcriptional activation in an improved whole-cell extract from *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **11**, 4555–4560.
25. Morris, D. P., Lee, J. M., Sterner, D. E., Brickey, W. J., and Greenleaf, A. L. (1997) Assaying CTD kinases in vitro and phosphorylation-modulated properties of RNA polymerase II in vivo. *Methods* **12**, 264–275.



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