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Phosphorylation of the Carboxy-Terminal Repeat Domain in RNA Polymerase II by Cyclin-Dependent Kinases Is Sufficient to Inhibit Transcription

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Abstract Cdc2 kinase triggers the entry of mammalian cells into mitosis, the only cell cycle phase in which transcription is globally repressed. We show here that Cdc2 kinase phosphorylates components of the RNA polymerase II transcription machinery including the RNA polymerase II carboxy-terminal repeat domain (CTD). To test specifically the effect of CTD phosphorylation by Cdc2 kinase, we used a yeast in vitro transcription extract that is dependent on exogenous RNA polymerase II that contains a CTD. Phosphorylation was carried out using immobilized Cdc2 so that the kinase could be removed from the phosphorylated polymerase. ATPγS and Cdc2 kinase were used to produce an RNA polymerase II0 that was not detectably dephosphorylated in the transcription extract. RNA polymerase II0 produced in this way was defective in promoter-dependent transcription, suggesting that phosphorylation of the CTD by Cdc2 kinase can mediate transcription repression during mitosis. In addition, we show that phosphorylation of pol II with the human TFIIH-associated kinase Cdk7 also decreases transcription activity despite a different pattern of CTD phosphorylation by this kinase. These results extend previous findings that RNA polymerase II0 is defective in preinitiation complex formation. Here we demonstrate that phosphorylation of the CTD by cyclin-dependent kinases with different phosphoryl acceptor specificities can inhibit transcription in a CTD-dependent transcription system. J. Cell. Biochem. 64:390–402.

Key words: carboxy-terminal repeat domain (CTD); RNA polymerase II; cyclin-dependent kinases; phosphorylation; transcription

Since the 1960s it has been known that transcription is shut off in mitosis [Prescott and Bender, 1962; Taylor, 1960], and recent in vitro experiments suggest that mitotic repression of transcription involves phosphorylation of components of the transcription machinery. For example, repression of transcription of RNA polymerase III–transcribed genes by Cdc2 kinase results from the direct phosphorylation of a component of the pol III transcription initiation factor TFIIIB [Gottesfeld et al., 1994; White et al., 1995]. We have previously shown that Cdc2 kinase phosphorylates the CTD of the large subunit of RNA polymerase II (pol II) [Cisek and Corden, 1989] and is able to destabilize the pol II preinitiation complex [Zawel et al., 1993]. Kinase was added directly to the transcription complex in this experiment, however, and it could not be determined whether disruption of the preinitiation complex was caused by phosphorylation of pol II or phosphorylation of other general transcription factors.

Phosphorylation of the CTD has been proposed to play an important role in the transcription cycle. Two forms of the largest subunit of pol II have been identified in cells: the unphosphorylated IIA form (pol IIA) and the phosphorylated II0 form (pol II0) which migrates more slowly in SDS polyacrylamide gels [Cadena and Dahmus, 1987]. The shifted II0 form contains a CTD that is phosphorylated at multiple sites [Cadena and Dahmus, 1987]. A number of kinases have been shown to phosphorylate the CTD in vitro, converting the IIA form to the II0

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form [Arias et al., 1991; Baskaran et al., 1993; Cisek and Corden, 1989, 1991; Dubois et al., 1994; Feaver et al., 1991; Guilfoyle, 1989; Lee and Greenleaf, 1989; Legagneux et al., 1990; Payne and Dahmus, 1993; Payne et al., 1989; Peterson et al., 1992; Serizawa et al., 1992; Stone and Reinberg, 1992]. These kinases differ in their specificity, recognizing different amino acid side chains and, in the case of serines, different positions within the heptapeptide repeat [Baskaran et al., 1993; Stone and Reinberg, 1992; Zhang and Corden, 1991]. While it is not clear which, if any, of these kinases are essential for CTD phosphorylation in vivo, CTD mutations that alter potential phosphorylation sites are lethal, indicating that CTD phosphorylation is essential [West and Corden, 1995].

In different cell-free transcription systems, pol II0 has been shown to be the predominant form present in elongation complexes [Bartholomew et al., 1986; Weeks et al., 1993], but pol IIA preferentially enters the preinitiation complex [Chesnut et al., 1992; Kang and Dahmus, 1993; Lu et al., 1991; Serizawa et al., 1993]. Phosphorylation of pol IIA in vitro can take place within the preinitiation complex or shortly after the transition to elongation [Laybourn and Dahmus, 1989, 1990; Payne et al., 1989], and some experiments indicate that phosphorylation of the CTD prevents the assembly of RNA polymerase II into preinitiation complexes [Chesnut et al., 1992; Kang and Dahmus, 1993; Lu et al., 1991; Serizawa et al., 1993]. These experiments have led to the hypothesis that pol IIa is a specialized form of pol II that is required for initiation, while the role of pol II0 is restricted to elongation [Dahmus, 1995].

The interpretation of experiments indicating that pol II0 is defective in initiation is complicated by limitations of the experimental design. First, in some cases the kinase or kinases used were semipurified fractions with other protein contaminants, including general transcription factors which may themselves have been targets for phosphorylation [Chesnut et al., 1992; Kang and Dahmus, 1993]. Second, the systems used did not always require the CTD for promoter-dependent transcription [Chesnut et al., 1992; Lu et al., 1991; Serizawa et al., 1993]. Finally, the specific CTD residues phosphorylated by these kinases have not been determined [Chesnut et al., 1992; Lu et al., 1991; Serizawa et al., 1993]. The recent demonstration that different anti-CTD monoclonal antibodies recognized different pol II0 subforms suggests that the different pol II0s are structurally and perhaps functionally nonequivalent [Bregman et al., 1995].

To avoid these potential problems, we phosphorylated purified pol II with immunoaffinity-purified kinases having known CTD target residues. The modified pol II was separated from the kinase and added to a CTD-dependent transcription extract prepared from yeast cells harboring an RNA polymerase with a truncated CTD consisting of nine consensus repeats (CTD9) [West and Corden, 1995]. Such extracts transcribe inefficiently but can be supplemented with purified wild-type pol II to restore transcription [Liao et al., 1991]. The CTD9 mutant strain grows nearly as well as wild-type but is temperature- and cold-sensitive. Transcription in the extract is stimulated by the addition of purified wild-type pol IIA but not by pol II lacking the CTD. We have used this CTD-dependent transcription system to show that pol II0 produced in vitro with either Cdc2 kinase or the TFIIH-associated kinase Cdk7 is unable to transcribe when dephosphorylation of the CTD is prevented. We also show that Cdc2 kinase and the TFIIH-associated Cdk7 kinase or its yeast homolog Kin28p have different specificities for phosphoryl group acceptors in the CTD.

MATERIALS AND METHODS

Reagents

Cdc2 kinase purified from the sea star *Pisaster ochraceus* was purchased from Upstate Biotechnology Incorporated (Lake Placid, NY). [α-^{32}P] UTP (800 Ci/mmol), [γ-^{32}P] ATP (3,000 Ci/mmol), and [^{125}I]-labeled goat-antimouse IgG secondary antibody were purchased from Dupont–New England Nuclear (Boston, MA). Protease-free BSA (fraction V) was obtained from Boehringer-Mannheim (Indianapolis, IN). Protease inhibitors were purchased from Sigma (St. Louis, MO) or Boehringer-Mannheim. T1 ribonuclease was obtained from Gibco/BRL (Gaithersburg, MD). Western blot ECL reagents were purchased from Amersham (Arlington Heights, IL). Affigel-10 was from Bio-Rad (Richmond, CA). Protein concentrations were determined with Bio-Rad protein assay reagent using BSA as a standard.

Yeast Strains

The *Saccharomyces cerevisiae* strain YX(9)-11 is a derivative of Z26 [Nonet et al., 1987] *MATa his3Δ200 ura3-52 leu2-3,112 rpb1Δ D187::HIS3 GAL*$^+$ (pRP112), in which the wild-type *RPB1* gene was replaced with a plasmid-borne *rpb1* allele encoding only nine repeats of the CTD [West and Corden, 1995].

Plasmids

Plasmid pMGL6 contains the yeast TEF1 promoter [Cottrelle et al., 1985] fused to a G minus cassette [Sawadogo and Roeder, 1985]. This plasmid was constructed as follows. pGAL4CG$^-$ [Lue et al., 1989] was used as a template to amplify by polymerase chain reaction (PCR) the G$^-$ region extending from 39 to 372 bases downstream of the initiation site. The G$^-$ fragment was then cloned into plasmid pUC18 via *Asp*718 and *Hin*cII/*Eco*RV sites in the PCR primers to form pLG$^-$. Yeast genomic DNA from the strain YH8 [Xu and Boeke, 1990] was used as the template to PCR-amplify the TEF1 promoter region from position −147 to −28 with respect to the translational start site. This region includes the TATA box and the start site of transcription. The start site was altered such that the G at position −24 was changed to an A. The TEF1 promoter was cloned into an *Eco*RV/*Pst*I fragment of pLG$^-$ to form pTLG$^-$. The TEF1 UAS extending from −469 (*Hin*dIII) to −131 (*Bam*HI) was PCR-amplified and inserted into a *Bam*HI/*Hin*dIII fragment of pTLG$^-$ to form pMGL6. This clone thus contains the full TEF1 promoter including the UAS and TATA box. Plasmid pMGL19 is similar to pGAL4CG$^-$ but has the GAL1/GAL10 UAS instead of just the Gal4p binding element, and the G at position −35 was not changed to a C. It was constructed as follows. The CYC1 promoter (CYC1 TATA and start site) from −138 to −35 with respect to the translational start site was PCR-amplified from genomic DNA of yeast strain YH8 and ligated into a *PstI*/*Eco*RV fragment of pLG$^-$ to form pCLG$^-$. The GAL1/GAL10 UAS from 288 to 663 was also obtained by PCR from genomic DNA of YH8 and cloned into the *Bam*HI/*Hin*dIII fragment of pCLG$^-$ to form pMGL19. All plasmid constructs were verified by sequence analysis.

Purification of RNA Polymerase II

RNA polymerase II was purified to homogeneity from the yeast *Saccharomyces cerevisiae* strain YCP50/TFB1.6HIS (a gift from the R. Kornberg lab) as described [Sayre et al., 1992] except that after the hydroxylapatite column the polymerase was purified on a DEAE-5PW column with a linear gradient from 0.2 to 0.9 M potassium acetate in buffer F (20 mM Tris acetate, pH 7.8, 20% glycerol, 0.25 mM EDTA, 1 mM dithiothreitol, 0.01% Nonidet P-40, potassium acetate, and protease inhibitors (1 mM PMSF, 2 μM Pepstatin, 2 μM chymostatin, 0.6 μM leupeptin, 2 mM benzamidine hydrochloride)). RNA polymerase II eluted at 0.65 M potassium acetate and was then dialyzed against 20 mM Tris-Cl, pH7.5, 0.1 M $(NH_4)_2SO_4$, 20% glycerol, 0.1 mM EDTA, 10 mM dithiothreitol, 0.01% Nonidet P-40, and protease inhibitors. Silver-stained gels showed that the pol II fraction was over 90% pure. Aliquots were stored at −80°C. Pol II with nine CTD repeats (CTD9) was purified from strain YX(9)-11 [West and Corden, 1995] as above. RNA polymerase II lacking the CTD [Li and Kornberg, 1994] was a gift from the R. Kornberg lab kindly provided by G. Meredith.

Preparation of Immobilized Cdc2 Kinase

Hemagglutinin (HA) epitope-tagged human Cdc2 and untagged human cyclin B1 were produced in Sf9 insect cells by infection with recombinant baculoviruses [Desai et al., 1992] (a gift from D.O. Morgan, UCSF). Lysates were prepared as in Desai et al. [1992] and were stored at −80°C at a concentration of 8 mg/ml total protein for cyclin B and 4 mg/ml Cdc2. Anti-HA epitope tag monoclonal antibody 12CA5 [Wilson et al., 1984] was prepared from mouse ascites fluid. After ammonium sulfate precipitation, IgG was purified by chromatography on hydroxylapatite and was bound to Affi-gel 10 (Bio-Rad) according to the manufacturer's instructions at a final concentration of 10–12 mg/ml. 12CA5 beads were prepared for binding to Cdc2 kinase by first washing three times in ten volumes of wash solution I (150 mM NaCl, 10 mM Tris, pH 7.8, 1 mM EDTA, 2.5 mg/ml protease-free BSA, 1 mM PMSF, 2 μM pepstatin, 0.6 μM leupeptin, 2 mM benzamidine HCl, 10 μg/ml bestatin). Crude baculovirus lysates containing Cdc2 and cyclin B1 were mixed

in a 1:1 ratio in the presence of 1 mM ATP for 20 min at 23°C and were then added to 12CA5 Affi-gel beads at a volumetric ratio of 3:1 (Cdc2 kinase:beads). After gentle rocking at 4°C for 1.25 h, unbound crude lysate was removed by centrifugation, and the beads were washed one time in ten volumes wash solution I (see above), three times in wash solution II (150 mM NaCl, 10 mM Tris, 1 mM EDTA, 0.1% Triton X-100 (Pierce, Rockford, IL), 2.5 mg/ml BSA, and protease inhibitors as above), and three times in wash solution IIIB (50 mM Tris-acetate, 50 mM potassium glutamate, 10 mM magnesium acetate, 2.5 mg/ml protease free BSA, and protease inhibitors as above).

Phosphorylation of RNA Polymerase II with Immobilized Cdc2 Kinase

For each phosphorylation reaction, 10 µl of Cdc2 kinase immobilized on 12CA5 Affi-gel beads was incubated with 10 µl of RNA polymerase II (~200 ng) in 60 mM potassium glutamate, 50 mM Tris-acetate, pH 7.8, 10 mM magnesium acetate, 0.5 mM DTT, 2.5 mg/ml protease-free BSA, protease inhibitors as above, and, in some experiments, phosphatase inhibitors (1.5 mM sodium azide, 1.5 mM NaF, 3 mM levamisole). The reaction was pulsed with 20 µCi of [γ-^{32}P] ATP for 5 min and then chased with 1 mM ATP (or water for unchased control) for 20 min at room temperature, with gentle rocking. The reaction with ATPγS was performed for 40 min (both unchased control and ATP control were also incubated for 40 min in these experiments). Phosphorylated RNA polymerase II was removed from the beads by spinning the supernatant through a BSA-treated filter (UFC3 OHV 00; Millipore, Bedford, MA). The level of phosphorylation was assessed by electrophoresis in a 5% polyacrylamide-SDS gel followed by direct autoradiography of the dried gel or by transfer to PVDF membrane and probing with the CTD-specific monoclonal antibody 8WG16 [Thompson et al., 1989]. Quantification of radioactivity was done with a Molecular Dynamics (Sunnyvale, CA) Phosphorimager.

Transcription Assay

Whole-cell extracts from *Saccharomyces cerevisiae* cells containing RNA polymerase II with only nine CTD repeats were made according to the method of Woontner et al. [1991]. Cells were grown in YPD, harvested at a density of 2–4 $\times$ 10^7 cells/ml, and disrupted using glass beads in a DYNO-MILL (Glen Mills Inc, Maywood, NJ). Transcription reactions were carried out as described in Woontner et al. [1991]. Briefly, 25–200 ng purified wild-type RNA polymerase II was added to the extract containing 5 mg/ml total protein in 50 mM Hepes-KOH, pH 7.5, 90 mM potassium glutamate, 10 mM magnesium acetate, 10% glycerol, 0.75% polyethylene glycol (M.W 3,350), 2.5 mM DTT, 0.5 units Inhibit-ACE (5 Prime–3 Prime, Boulder, CO), 10 µg/ml DNA template, 0.4 mM CTP, 0.4 mM ATP, 2 µM [α-^{32}P] UTP at 10^6 cpm/pmole, 30 mM creatine phosphate, 1,400 units/ml creatine kinase, and 2 units RNase T1. The template and nucleotides were added last. Reaction time was 20 or 30 min at 23°C. The reactions were stopped and processed as described [Woontner et al., 1991], and transcripts were run on 4% acrylamide/urea gels. Assays for the capability of the phosphorylated form of RNA polymerase II to transcribe were done under conditions where exogenous pol IIA stimulates transcription twenty- to seventyfold above the background of the defective CTD9 extract.

Addition of Purified Cdc2 Kinase to Reconstituted Transcription Reactions

Transcription reactions reconstituted from purified components were as previously described [Sayre et al., 1992] using 100–200 ng purified RNA polymerase II, 24 ng yeast recombinant TBP, 3 ng purified yeast TFIIE, 24 ng recombinant TFIIB, 2 µl MonoS fraction of yeast TFIIH, 50 ng purified yeast TFIIF, and 10 µg/ml template (pMGL19). Purified sea star Cdc2 kinase (15 ng) was added to the mix of pol II and factors; template and nucleotides were added last. Reactions were incubated at 23°C for 30 min. Reaction products were labeled with [α-^{32}P] UTP for transcription analysis, or with 20 µCi [γ-^{32}P] ATP if they were to be assayed for phosphorylation.

Phosphorylation of RNA Polymerase II With Immobilized CAK

Purified human cyclin-dependent kinase activating kinase CAK (0.4 mg/ml), a generous gift from David Morgan (UCSF), is comprised of HA-tagged MO15/CDK7, cyclin H, and p36, all of which were derived from baculovirus infected cells. It was purified on protein A beads as in Fisher and Morgan [1994] to about 90% purity. 12CA5 Affi-gel beads were prepared for

binding by first washing four times in ten volumes of HBSB (10 mM Hepes-KOH, pH 7.5, 150 mM NaCl, 2.5 mg/ml protease free BSA, and protease inhibitors as above). Approximately 2 µg of HA-tagged CAK was added in 30 µl of HBSB to 10 µl of 12CA5 beads, and the mixture was rocked at 4°C for 75 min. Unbound CAK was then removed, and the beads were washed three times in ten volumes of HDB (25 mM Hepes, 1 mM DTT, 2.5 mg/ml protease-free BSA, and protease inhibitors as before).

For each phosphorylation reaction, 10 µl of CAK immobilized on 12CA5 Affi-gel beads was incubated with 10 µl of RNA polymerase II (100–200 ng) in CAK buffer. The reaction was pulsed for 5–10 min with 20 µCi [γ-^{32}P] ATP and then chased for 40 min with 1 mM ATP or 1 mM ATPγS at room temperature. Phosphorylated polymerase was removed from the beads by ultrafiltration and assayed as described for Cdc2 kinase–phosphorylated pol II.

Determination of the Specificity of Cdc2 Kinase and CAK Using Peptide Substrates

Peptides containing eight heptapeptide repeats were synthesized using T-BOC chemistry. The sequences of the three peptides used are WT = $(SPTSPSY)_8$, A2 = $(\underline{A}PTSPSY)_8$, A5 = $(SPT\underline{A}PSY)_8$, JC13 = SP$\overline{T}$TPR YTPQSPT YSPT$\overline{S}$PV YSPSSPNY, and JC14 = SPTSPR YTPQSPT YTPVSPS YSPSSPAY. JC13 and JC14 are based on nonconsensus repeats 31–34 of the mouse CTD with additional amino acid changes corresponding to substitutions present in other nonconsensus repeats. Peptides were purified by reverse phase chromatography, lyophilized to dryness, and resuspended in water. Each kinase reaction contained 4 µg peptide in 10 µl 50 mM Tris, pH 7.9, 100 mM KCl, 10 mM $MgCl_2$, and 10 µCi [γ-^{32}P] ATP. Reactions were initiated by addition of kinase (1 µl of sea star Cdc2 kinase or 2 µl of human CAK) and were incubated at 23°C for 30 min. An equal volume of 2× SDS sample buffer was added, and the samples were electrophoresed on a 12.5% polyacrylamide-SDS gel. The wet gel was exposed directly for autoradiography. For phosphoamino acid analysis, peptides were labeled in reactions containing 6 µg of peptide, 15 µCi [γ-^{32}P] ATP, and either immobilized Cdc2 kinase derived from baculovirus infected cells as described above or yeast TFIIH-associated kinase [Sayre et al., 1992]. Labeled peptides were run on an SDS gel and blotted to an immobilon filter, and phosphoamino acids were analyzed as described [Kamps and Sefton, 1989].

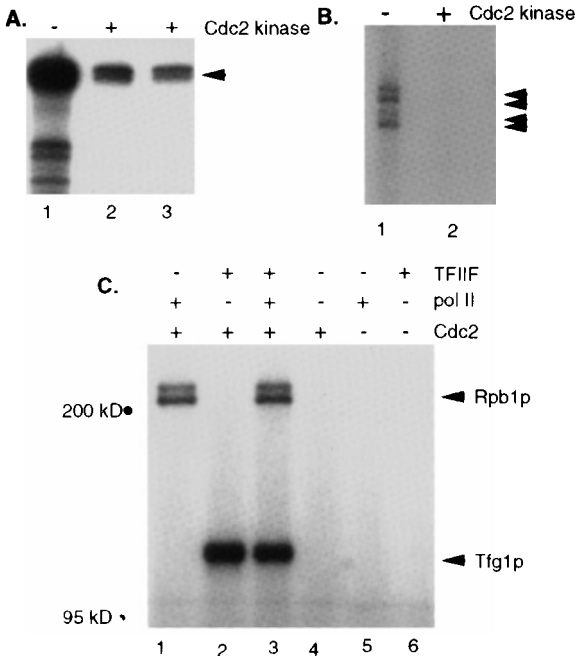

Fig. 1. Cdc2 kinase inhibits pol II transcription in crude and purified yeast systems. **A:** Full-length transcripts (arrowhead) made in whole-cell extract from CTD9 cells (Materials and Methods) supplemented with 100 ng of purified wild-type pol IIA and pMGL19. *Lane 1:* In the absence of Cdc2 kinase. *Lanes 2, 3:* In the presence of 15 ng of purified sea star Cdc2 kinase. Samples were preincubated with Cdc2 kinase for 20 min at 4°C (lane 2) or 23°C (lane 3). **B:** Transcripts (arrowheads) made with purified factors (Materials and Methods). *Lanes 1, 2:* Transcripts from pMGL19 in the absence or presence of Cdc2 kinase, respectively. **C:** Pure pol II and/or TFIIF was incubated with [γ^{32}-P] ATP and Cdc2 kinase as indicated, and resulting ^{32}P-labeled proteins were separated on a SDS-5% polyacrylamide gel and visualized by autoradiography. Rpb1p is the largest subunit of RNA polymerase II while Tfg1p is the largest subunit of TFIIF. Reactions did not contain template.

RESULTS

Cdc2 Kinase Inhibits Transcription in Crude and Purified Yeast Systems

We previously reported that Cdc2 kinase phosphorylates the CTD of RNA polymerase II [Cisek and Corden, 1989, 1991] and that addition of Cdc2 kinase to a HeLa-derived transcription reaction destabilizes the preinitiation complex [Zawel et al., 1993]. We show here that purified Cdc2 kinase inhibits transcription in a yeast whole cell extract (Fig. 1A, lanes 2, 3) or in a purified transcription system reconstituted with pol II and the basal transcription factors TBP, TFIIB, TFIIE, TFIIF, and TFIIH (Fig. 1B, lane 2). Because the CTD is dispensible in this

reconstituted system [Li and Kornberg, 1994], inhibition of the reconstituted system suggests that components of the basal transcription machinery other than the CTD could be targets of Cdc2 kinase.

Incubation of Cdc2 kinase with individual components of the basal transcription machinery in the presence of [γ-^{32}P] ATP identified several potential targets. The most reactive substrates were the large subunit of RNA polymerase II (Fig. 1C, lanes 1, 3) and the large subunit of TFIIF (Fig. 1C, lanes 2, 3). Pol II alone did not give rise to a phosphorylated band at the position of the TFIIF subunit (lane 1), indicating that our pol II is not contaminated with TFIIF (note that the presence of pol II did not inhibit phosphorylation of Tfg1p [lane 3]). To determine specifically whether pol II was inactivated by phosphorylation, we performed the experiments described below.

Transcription in a CTD-Dependent System

To test the effect of CTD phosphorylation on transcription, we developed a CTD-dependent in vitro transcription system. Previous studies showed that crude yeast extracts require the CTD for transcription, while a reconstituted system does not [Li and Kornberg, 1994; Liao et al., 1991]. It was thus necessary to use a crude extract dependent on exogenous pol II. Liao et al. [1991] showed that in vitro transcription is deficient in extracts of mutant cells containing pol II with a truncated CTD. We made extracts from yeast cells containing a mutant pol II in which the CTD has only nine heptapeptide repeats (CTD9) [West and Corden, 1995]. This strain is viable, yet whole-cell extracts showed low levels of transcription with all G$^-$ templates tested (Fig. 2A, lane 1; data not shown). Addition of purified pol II to CTD9 whole-cell extract stimulated transcription activity over fiftyfold (Fig. 2A, lanes 5, 6). This stimulation did not occur when purified CTD-less pol II (Fig. 2A, lanes 2–4) or pol II purified from the CTD9 strain (Fig. 2B) was added to the extract (purified CTD-less pol II transcribes as well as wild-type pol II in a purified reconstituted system [Li and Kornberg, 1994]). In addition, anti-CTD monoclonal antibody 8WG16 [Thompson et al., 1989] inhibited transcription in our crude extract (Fig. 2C, compare lanes 3 and 4), yet this antibody does not inhibit transcription in a purified system [Conaway et al., 1992]. Thus, unlike the reconstituted system, transcription

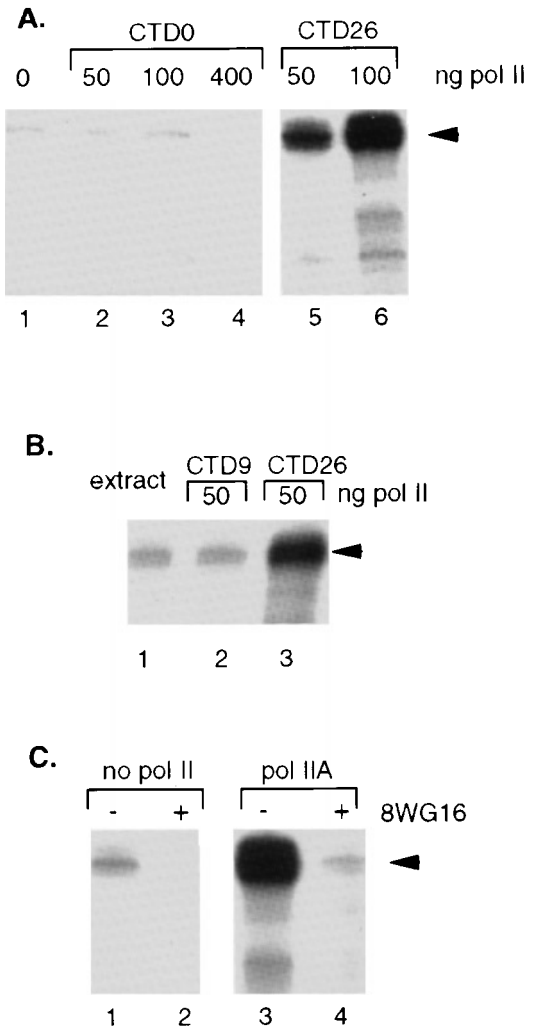

Fig. 2. Transcription in CTD9 extract is CTD-dependent. **A:** *Lane 1:* Transcripts (arrowhead) from pMLG6 template in the CTD9 extract. *Lanes 2–4:* Transcripts from extract supplemented with purified CTD-less polymerase (CTD0). *Lanes 5, 6:* Transcripts in extract supplemented with purified wild-type polymerase (CTD26). **B:** *Lane 1:* Transcript (arrowhead) from pMLG6 template in the CTD9 extract. *Lane 2:* Transcript from extract supplemented with purified RNA polymerase containing nine repeats (CTD9). *Lane 3:* Transcript in extract supplemented with purified wild-type polymerase (CTD26). **C:** Addition of purified monoclonal anti-CTD antibody 8WG16 to CTD9 extracts inhibits transcription. *Lanes 1, 2:* Reactions contained no added pol II and antibody (2 µg) as indicated. *Lanes 3, 4:* Reactions supplemented with wild-type pol II (170 ng) and antibody as indicated.

in the CTD9 extract depends on exogenous pol II with an intact CTD.

Phosphorylation of the IIA Form of RNA Polymerase II by Cdc2 Kinase Quantitatively Converts It to the II0 Form

We used an HA-tagged Cdc2 kinase immobilized on anti-HA Affi-gel beads to phosphorylate

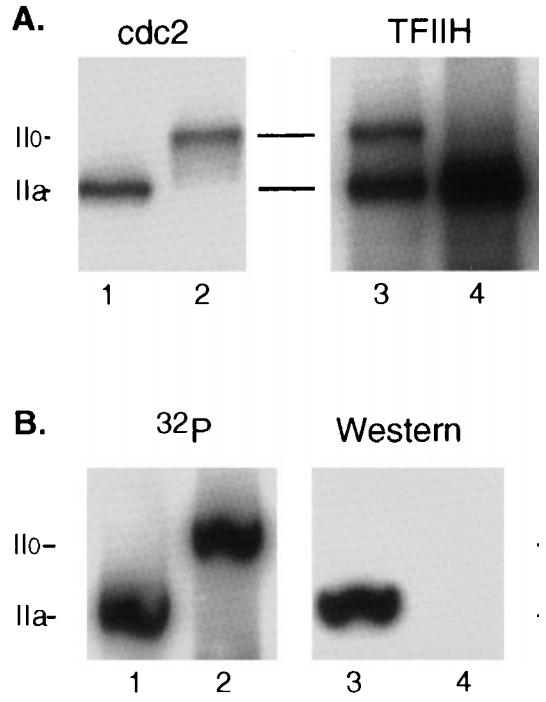

Fig. 3. Cdc2 kinase hyperphosphorylates Pol II to produce a slower mobility (II0) form. Phosphorylated and unphosphorylated pol II were separated by SDS-PAGE (Materials and Methods) and detected either by autoradiography (A,B) or by Western Blot (B). **A:** *Lanes 1, 2:* Pol II was pulsed with 20 μCi [γ-^{32}P] ATP for 5 min and run on SDS-PAGE directly or chased with 1 mM ATP prior to electrophoresis, respectively. *Lanes 4, 3:* Pol II was pulsed with 20 μCi [γ-^{32}P] ATP for 5 min and run on SDS-PAGE or chased with 1 mM ATP prior to electrophoresis, respectively. **B:** Immobilized Cdc2 kinase (Materials and Methods) was used to phosphorylate pol II. *Lanes 1, 2:* Reactions pulsed with 20 μCi [γ-^{32}P] ATP for 5 min and chased with 1 mM ATP for 20 min, respectively. Labeled proteins were separated on a 5% polyacrylamide-SDS gel and visualized by autoradiography. *Lanes 3, 4:* Untreated pol II and pol II phosphorylated with Cdc2 kinase in the presence of nonradiolabeled ATP, respectively. Samples were subjected to Western blotting using monoclonal antibody 8WG16 (Materials and Methods).

pol II (Materials and Methods). Phosphorylation was monitored by observing a shift to the II0 form in SDS-polyacrylamide gels (Fig. 3A, lane 2). This shift is the same as that obtained when pol II was phosphorylated with yeast TFIIH (Fig. 3A, lane 3). Phosphorylated polymerase was removed from the kinase beads for the purpose of testing its transcription capability. No residual kinase was detected in the phosphorylated pol II prepared in this way.

To ensure that phosphorylation of pol II by Cdc2 kinase was complete, we subjected the phosphorylated enzyme to Western blotting using anti-CTD mAb 8WG16. 8WG16 is able to detect the nonphosphorylated IIA form but not the phosphorylated or II0 species [Zhang and Corden, unpublished observations]. Indeed, as can be seen in lane 3 of Figure 3B, there is an antibody-reactive band where the IIA form migrates, while no bands are seen in the II0 lane (Fig. 3, lane 4). This result is not due to a transfer problem, as the ^{32}P-labeled II0 form in the same gel transferred well (Fig. 3B, lane 2). Thus, virtually all of the IIA form was shifted to the II0 form with Cdc2 kinase.

Transcription Activity of Phosphorylated RNA Polymerase II in a CTD-Dependent System

Pol II was converted to the II0 form with Cdc2 kinase as described above, separated from the kinase, and either subjected to electrophoretic analysis or added to the CTD9 extract to test its activity. Surprisingly, quantitative phosphorylation did not block transcription under these conditions (see Fig. 6 and below); quantification of transcription gels and of the relative amounts of pol II0 and IIA revealed only a slight (10–50%) decrease in transcription for the II0 form (data not shown). Thus, pol II0 appeared able to transcribe almost as well as the IIA form when added to a CTD9 extract regardless of the promoter used (pGAL4CG$^-$, pMGL6, or pMGL19; Materials and Methods; data not shown).

This result prompted us to assess spontaneous dephosphorylation of pol II0 in the CTD9 extract. Both the IIA and II0 forms were incubated under transcription conditions, and samples were taken at 0 and 20 min for protein analysis by SDS-PAGE. Autoradiography and Western blotting showed that up to 40% of the input pol II was indeed dephosphorylated within 20 min (data not shown).

To determine if transcription by the II0 form was enabled by this dephosphorylation, the kinase reaction was repeated in the presence of ATPγS instead of ATP to prevent dephosphorylation [Tazi et al., 1993]. Pol II was phosphorylated with ATP or ATPγS to produce II0 (Fig. 4A, compare lanes 2 and 3). No significant dephosphorylation was observed after incubation of thiophosphorylated pol II under transcription conditions (Fig. 4A, compare lanes 5 and 6). In contrast to ATP-phosphorylated pol II, the thiophosphorylated protein was severely impaired in its ability to stimulate transcription in a CTD9 extract (Fig. 4B, compare lanes 3, 4, and 8 with 5 and 6). Mixing both IIA and

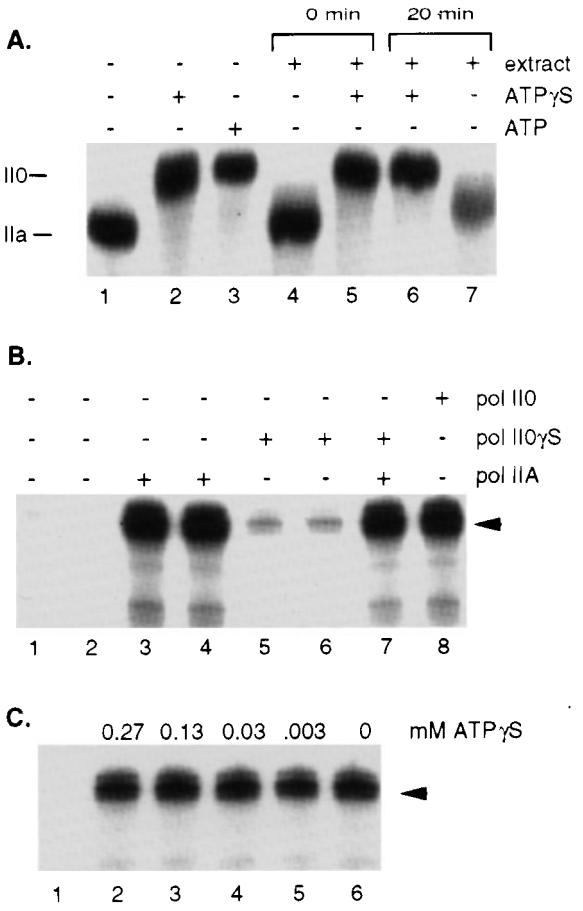

Fig. 4. Phosphorylation of Pol II with ATPγS prevents transcription by IIO. **A:** Formation of pol IIO with Cdc2 kinase (Materials and Methods). *Lanes 1–3:* IIA, IIO (ATPγS-phosphorylated), and IIO (ATP-phosphorylated). *Lanes 4–7:* IIA and IIO (ATPγS) incubated under transcription conditions for indicated times. **B:** Transcripts from pMGL6 (arrowhead) in CTD9 extract (*lanes 1, 2*) supplemented with pol IIA (*lanes 3, 4*) or thiophosphorylated Pol IIO (*lanes 5, 6*). *Lane 7:* Pol IIA (40 ng) and 40 ng of pol IIOγS. *Lane 8:* ATP-phosphorylated pol IIO. Approximately 80 ng of the pol IIs shown in A were used in lanes 3–8. **C:** Transcripts (arrowhead) obtained in the presence of ATPγS and 0.4 mM ATP. *Lane 1:* CTD9 extract alone. *Lanes 2–6:* Pol IIA and ATPγS as indicated.

thiophosphorylated IIO forms in the same reaction did not inhibit transcription (Fig. 4B, lane 7), indicating that thiophosphorylated IIO does not block transcription by IIA in trans and that no other trans-acting inhibitory byproducts are generated in the thiophosphorylation reaction. Moreover, titration of ATPγS directly into the CTD9 extract supplemented with wild-type pol IIA had no effect on transcription (Fig. 4C), ruling out the possibility that residual ATPγS in the thiophosphorylated pol IIO sample accounted for the observed inhibition.

Cdc2 Kinase and CAK Phosphorylate the CTD on Different Sites

Pol IIO has been shown to be less efficient than pol IIA in preinitiation complex formation on the adenovirus-2 major late promoter [Chesnut et al., 1992; Lu et al., 1991], and our results extend that observation to transcription from a variety of yeast promoters. Since many other kinases phosphorylate the CTD, we wanted to know if the inactivity of pol IIO in promoter-dependent transcription depends on the kinase employed or is a general property of pol IIO. We previously found that Cdc2 kinase phosphorylates the CTD at both serines 2 and 5 of the consensus heptapeptide $Y_1S_2P_3T_4S_5P_6S_7$ [Zhang and Corden, 1991]. We show here that another kinase implicated in CTD phosphorylation, the human TFIIH-associated CTD kinase CAK [Roy et al., 1994], which produces pol IIO with the same mobility as Cdc2 kinase–phosphorylated pol II (Fig. 3A; see below), nevertheless has a different substrate specificity. We used three different synthetic CTD peptides containing eight heptapeptide repeats as kinase substrates (Fig. 5A; Materials and Methods). These three peptides differ in that the serines shown to be phosphorylated by Cdc2 kinase [Zhang and Corden, 1991] (positions two and five) are substituted with alanine, thus preventing phosphorylation at that position. Figure 5B shows that while Cdc2 kinase is able to phosphorylate all three synthetic peptides, both partially purified yeast TFIIH (MonoS fraction [Sayre et al., 1992]) (Fig. 5B) and purified human TFIIH-associated kinase (CAK) (Fig. 5C) failed to phosphorylate position five–substituted peptide (A5) but are able to phosphorylate position two–substituted peptide (A2). Phosphoamino acid analysis of peptides with threonine substitutions at positions two and five (JC13) or two (JC14) confirmed that TFIIH kinase phosphorylates position five but not position two (Fig. 5D). Thus, although Cdk7 and Cdc2 catalytic subunits are related, they display a marked difference in their ability to phosphorylate the serine (or threonine) in position two of the consensus heptapeptide repeat.

Hyperphosphorylation of Pol II With CAK Inhibits Transcription

Though Cdc2 and CAK have different CTD substrate specificites, they both shift the largest subunit of pol II to the IIO form. To deter-

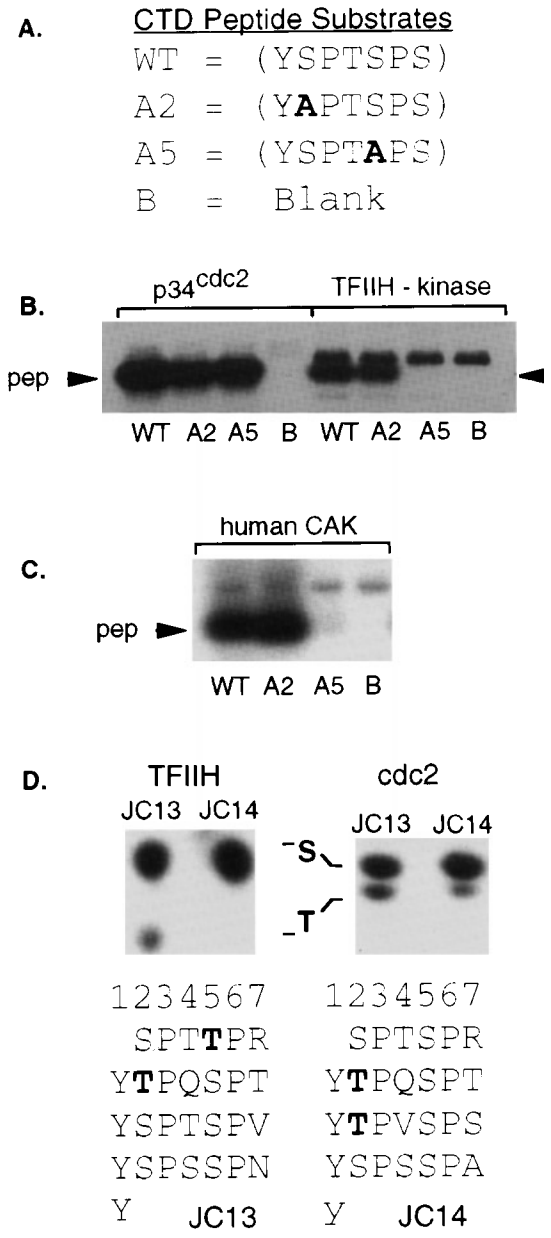

Fig. 5. Substrate specificity of Cdc2 and CAK. **A:** Substrate peptides (Materials and Methods). **B:** Autoradiograph of indicated peptides labeled with either Cdc2 kinase or yeast TFIIH-associated kinase, run on a 12.5% SDS polyacrylamide gel, and visualized by autoradiography. **C:** Peptides labeled with human CAK and separated as in B. **D:** Substrate specificities of TFIIH kinase and Cdc2 kinase tested on peptides containing threonine at postions two or five. Autoradiograph of one-dimensional separation of phosphoamino acids derived from peptides JC13 and JC14 (sequences shown below). Threonines in positions two and five (numbers above) are indicated by bold letters.

mine whether CAK-phosphorylated pol II0 is capable of transcribing, HA-tagged CAK bound to Affi-gel beads was used to phosphorylate pol IIA. The resulting pol II0 was then separated

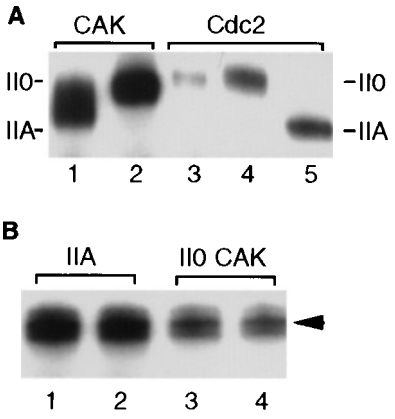

Fig. 6. Hyperphosphorylation of Pol II with CAK inhibits transcription. **A:** Phosphorylation of pol II with immobilized HA-tagged CAK. *Lanes 1, 2:* Pol II pulse-labeled with [γ-^{32}P] ATP for 8 min and chased for 40 min with ATPγS and ATP, respectively. *Lanes 3–5:* Cdc2 phosphorylated Rpb1p for comparison. Lane 5: Pulse-labeled pol IIA. Lanes 3, 4: Chased with ATP and ATPγS, respectively. **B:** Transcript (arrowhead) of pMGL6 in CTD9 extract supplemented with Pol II (untreated) (lane 1), pulse-labeled pol IIA (*lane 2*), CAK-phosphorylated pol II (A lanes 1, 2, respectively) (*lanes 3, 4*).

from the beads and assayed for activity in the CTD9 extract. Figure 6A (lane 2) shows that phosphorylation of pol II by CAK in the presence of ATP yields a shifted II0 form. Although we were not able to get a complete shift with ATPγS (Fig. 6A, lane 1), the results of Figure 6B nevertheless show that phosphorylation of pol II by CAK either with ATP (lane 4) or ATPγS (lane 3) decreases transcription activity. Thus, even though the shift was not complete with ATPγS-phosphorylated pol II, CAK-phosphorylated pol II transcribed poorly compared to unphosphorylated pol II, analogous to results obtained with Cdc2 kinase.

DISCUSSION

Phosphorylation of the CTD is viewed as an essential step in transcription initiation on the basis of findings indicating that (1) the CTD of RNA polymerases actively engaged in transcription is hyperphosphorylated (pol II0 [Bartholomew et al., 1986; Cadena and Dahmus, 1987; Payne et al., 1989; Weeks et al., 1993]), (2) hypophosphorylated polymerase molecules (IIA) are preferentially recruited to stable preinitiation complexes where they are subsequently phosphorylated [Chesnut et al., 1992; Kang and Dahmus, 1993; Laybourn and Dahmus, 1989, 1990; Lu et al., 1991, 1992; Payne and Dahmus, 1993; Payne et al., 1989; Serizawa et al., 1993], and (3) the basal transcription factor TFIIH

possesses a CTD kinase [Feaver et al., 1991; Lu et al., 1992; Serizawa et al., 1992] that is essential for transcription [Cismowski et al., 1995; Svejstrup et al., 1995; Valay et al., 1995]. In this model, pol IIA enters the preinitiation complex, where it makes contact with the general initiation factors. Subsequent phosphorylation of the CTD by TFIIH may trigger release of the polymerase to allow processive RNA chain elongation [Dahmus, 1995]. A hallmark of this model is the failure of pol II0 to assemble into preinitiation complexes in vitro as monitored by size exclusion chromatography and nondenaturing gel electrophoresis of DNA-protein complexes [Chesnut et al., 1992; Lu et al., 1991]. In addition, purified Cdc2 kinase added to a preinitiation complex can convert the polIA therein to pol II0 and disrupt the complex [Zawel et al., 1993].

Interpretation of such results in the context of the transcription mechanism is not straightforward, however. First, it is unclear whether the observed complexes are bona fide intermediates in the formation of active complexes since the transcription activity (when checked) is at least an order of magnitude lower than the efficiency of observed complex formation. Second, effects on complex stability might reflect phosphorylation of other transcription factors. The crude CTD-kinase fraction used by Dahmus and colleagues contained TFIIB, TFIIE, and TFIIF [Chesnut et al., 1992] and possibly multiple kinase activities. As we show here, Cdc2 kinase phosphorylates TFIIF. Since TFIIF mediates assembly of pol II into the preinitiation complex [Buratowski et al., 1991; Conaway et al., 1991; Ha et al., 1993], it is crucial to study the specific consequences of CTD phosphorylation under conditions where the phosphorylation state of other transcription factors is not influenced. Third, in previous experiments the specificities of the CTD kinases were not determined, leaving open the possibility that different patterns of CTD phosphorylation could differentially alter transcription. Finally, in purified transcription systems where phosphorylation of individual proteins can be controlled, initiation is independent of the CTD [Li and Kornberg, 1994; Serizawa et al., 1993].

The experiments reported here were designed to avoid these problems. We used a CTD-dependent transcription system in conjunction with well-characterized kinases (Cdc2 and CAK) and pol II that was phosphorylated apart from the transcription machinery and purified away from the kinase. Our results show that both kinases can convert pol IIA to pol II0. The activity of pol II0 in our CTD-dependent transcription system correlated with spontaneous dephosphorylation of the CTD in the extract. Dephosphorylation is not rate-limiting since time courses of transcription by pol IIA and Cdc2-phosphorylated pol II0 were virtually identical (data not shown). In contrast, thiophosphorylated pol II0 was severely reduced in its capacity to complement the CTD-dependent extract and was not detectably dephosphorylated during the reaction. These findings complement the binding studies of Dahmus and colleagues [Chesnut et al., 1992] and strengthen and extend the model for the regulatory role of CTD phosphorylation in the transcription cycle. We have shown in a novel and direct way that hyperphosphorylation of the CTD by cyclin-dependent kinases prior to transcription complex assembly can block transcription in a CTD-dependent yeast system and that this effect is due solely to the action of the kinase on the polymerase.

Cdc2 kinase phosphorylates serine residues 2 and 5 in the consensus CTD heptapeptide repeat. The related TFIIH-associated kinase from yeast and human cells phosphorylates only position five. Despite this different substrate specificity, all three kinases convert pol IIA to a II0 form that migrates more slowly in SDS-polyacrylamide gel electrophoresis. Phosphorylation of CTD tyrosine side chains by c-abl kinase also generates pol II0 [Baskaran et al., 1993]. It is therefore clear that pol II0 purified from cell extracts can consist of a mixture of qualitatively different enzymes [Bregman et al., 1995]. Endogenous Pol IIA purified from HeLa cells enters preinitiation complexes only four times more efficiently than does endogenous pol II0 [Lu et al., 1991]. Given this moderate degree of selectivity and the abundance of pol II0 in cells [Kim and Dahmus, 1986], the possibility that at least some forms of pol II0 initiate transcription in vivo cannot be ruled out. Comparative studies of different pol II0 forms are needed to determine whether they have distinct properties. In this first attempt along those lines, we found that the different pol II0s produced by Cdc2 and CAK are both defective in transcription. Whether the block is at initiation or a later step in the transcription cycle cannot be determined from the present

data. Whether the CTD becomes phosphorylated outside of the preinitiation complex in vivo is not known. A number of Cdc2-related CTD kinases have been found [Cisek and Corden, 1989, 1991; Feaver et al., 1994; Lee and Greenleaf, 1989, 1991; Liao et al., 1995; Roy et al., 1994; Shiekhattar et al., 1995], including those in a newly described pol II "holoenzyme" complex [Liao et al., 1995]. Not all of these kinases are found in the preinitiation complex, however. CTK1 kinase is not found in any of the general transcription factors, but mutations that inactivate this kinase result in a reduction (but not elimination) of CTD phosphorylation in vivo [Lee and Greenleaf, 1991]. This observation indicates that more than one kinase acts on the CTD in vivo and suggests that at least one of these kinases is not associated with the general factors involved in transcription initiation. Two types of regulatory mechanisms might control CTD phosphorylation by kinases. First, the accessibility of the CTD to phosphorylation might be regulated by controlling the association of the kinase with the transcription machinery, as appears to be the case with the TFIIH-associated kinase. Alternatively, the kinase may be regulated through cell-cycle activation, as for Cdc2. Finally, the role(s) of novel CTD phosphatases in these processes will also need to be addressed [Chambers and Dahmus, 1994; Chambers et al., 1995].

The in vitro experiments we present here suggest that the CTD is a target for kinases that negatively regulate transcription. Cdc2 kinase activity is increased in M phase and could lead to hyperphosphorylation of the CTD and global transcription repression. In preliminary immunoblotting experiments with an antibody against a non-CTD epitope of the largest subunit, we observed an increase in the abundance of II0 during mitosis [Gebara and Corden, unpublished observations]. Although we show here that Cdc2 kinase inhibits pol II transcription in vitro by phosphorylating the CTD, it remains to be shown whether this is sufficient for mitotic repression or if additional transcription factors such as TFIIF are targeted for inactivation in vivo. The approach we have developed here should be of general use in studying the potential transcription regulatory function of a wide variety of kinases.

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