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A carboxyl-terminal-domain kinase associated with RNA polymerase II transcription factor δ from rat liver

(mRNA synthesis/protein kinase/heptapeptide repeat)

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ABSTRACT **We previously purified RNA polymerase II transcription factor δ from rat liver and found that it has an associated DNA-dependent ATPase (dATPase) activity. In this report, we show that δ is also closely associated with a protein kinase activity that catalyzes phosphorylation of the largest subunit of RNA polymerase II. Kinase activity copurifies with transcription and DNA-dependent ATPase (dATPase) activities when δ is analyzed by anion- and cation-exchange HPLC as well as by sucrose gradient sedimentation, arguing that δ possesses all three activities. Phosphorylation of the largest subunits of both rat and yeast RNA polymerase II is stimulated by DNA, whereas phosphorylation of a synthetic peptide containing multiple copies of the carboxyl-terminal heptapeptide repeat is not. Although both ATP and GTP appear to function as phosphate donors, GTP is utilized less than 10% as well as ATP. These findings suggest that δ may exert its action in transcription at least in part through a mechanism involving phosphorylation of the largest subunit of RNA polymerase II.**

Promoter-specific transcription initiation by RNA polymerase II is preceded by the assembly of polymerase and at least five initiation factors into a complete, but inactive, preinitiation complex (1, 2). Conversion of the complete preinitiation complex to an active form, capable of initiating transcription, requires the action of an ATP (dATP) cofactor containing a hydrolyzable β-γ phosphoanhydride bond (3–5). Although the precise mechanism by which ATP activates the preinitiation complex is not known, recent evidence suggests that ATP hydrolysis drives unwinding of the DNA template at the site of transcription initiation to facilitate formation of an open complex (6).

An additional role for ATP in promoter-specific transcription has been investigated in light of evidence that phosphorylation of the carboxyl-terminal domain (CTD) of the largest subunit of RNA polymerase II may play a role in this process. The CTD is composed of as many as 52 tandem copies of the consensus sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser, which is extensively phosphorylated *in vivo* (7, 8). UV crosslinking studies indicate that, in isolated nuclei as well as in crude transcription extracts, the CTDs of polymerase molecules actively engaged in transcription are highly phosphorylated (8–10). Recent studies suggest, however, that at least *in vitro*, only the nonphosphorylated form of RNA polymerase II enters the preinitiation complex (11, 12). These results are consistent with the model that phosphorylation of the CTD is involved in conversion of the preinitiation complex from an inactive to an active conformation or, as proposed by Dahmus and colleagues (10, 13) and by Sigler (14), in the transition from an initiation to an elongation complex. Nonetheless, the interpretation of these results is complicated by the observation that RNA polymerase II lacking all or most of the CTD is capable of catalyzing accurate initiation from some promoters *in vitro* (15–18). Furthermore, it is clear that phosphorylation of the CTD is not the sole function of ATP, since accurate initiation by polymerase lacking this domain still requires an ATP cofactor (19).

Protein kinases capable of phosphorylating the CTD have been identified in a variety of cells. Two CTD kinases containing a homolog of $p34^{cdc2/CDC28}$ and either 58-kDa or 62-kDa subunits have been purified from mouse cells (20, 21). In addition, a CTD kinase composed of 58-, 38-, and 32-kDa polypeptides has been purified from yeast (22, 23). The 58-kDa subunit of this enzyme exhibits substantial sequence similarity with the cdc2/CDC28 family of protein kinases, and mutations that disrupt the gene encoding this subunit dramatically reduce, but do not abolish, phosphorylation of the largest subunit of RNA polymerase II *in vivo*.

In addition, several laboratories have identified template-associated (19, 24) or DNA-stimulated (25) protein kinases that are capable of phosphorylating the CTD in the preinitiation complex, either immediately before or during initiation. However, a requirement for these kinases in transcription has not been demonstrated, nor has it been shown that either kinase is associated with any of the known RNA polymerase II transcription factors. A direct demonstration that a transcription factor required for initiation by RNA polymerase is capable of catalyzing phosphorylation of the CTD was lacking until recently, when Feaver *et al.* (26) reported that purified transcription factor b from yeast is associated with both DNA-dependent ATPase and CTD kinase activities. Factor b is one of several transcription factors required for promoter-specific initiation in a reconstituted transcription system from yeast (27, 28).

We previously identified and purified a mammalian transcription factor, designated δ, which possesses an associated DNA-dependent ATPase (dATPase) activity (29). δ, along with additional transcription factors, is required for initiation by RNA polymerase II from the core regions of many TATA-box-containing promoters in a reconstituted transcription system derived from rat liver (30). In this report, we present evidence that transcription factor δ, like yeast factor b, also possesses a closely associated protein kinase activity capable of catalyzing phosphorylation of the largest subunit of RNA polymerase II.

MATERIALS AND METHODS

Materials. Unlabeled ultrapure ribonucleoside 5′-triphosphates and 2′-deoxynucleoside 5′-triphosphates were purchased from Pharmacia LKB. [γ-^{32}P]ATP (>4000 Ci/mmol; 1 Ci = 37 GBq) was from ICN or Amersham. [α-^{32}P]CTP (>800 Ci/mmol) and [α-^{32}P]dATP (>800 Ci/mmol) were from ICN, and [γ-^{32}P]GTP (≈5000 Ci/mmol) was from Amersham.

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Abbreviation: CTD, carboxyl-terminal domain.

Preparation of RNA Polymerase II and Transcription Factors. Transcription factors α (31), $\beta\gamma$ (32), δ (29, 33), and ε (34) were purified from rat liver as described. Recombinant yeast TFIID was expressed and purified as described (34) from bacterial strain N5151 containing the plasmid pASY2D (35). RNA polymerase II containing the IIa form of the largest subunit was purified from rat liver nuclear extracts as described (36), except that Ultrogel AcA 34 (IBF) and Spherogel TSK DEAE-5-PW (Beckman) were used instead of 4000SW Spherogel TSK and TSK DEAE-NPR, respectively.

Assay of Protein Kinase. Except as indicated in the figure legends, reaction mixtures (15 μl) were incubated for 1 hr at 28°C and contained 3 mM Hepes/NaOH (pH 7.9), 20 mM Tris/HCl (pH 7.9), 2% polyvinyl alcohol, 7.5 μg of bovine serum albumin, 60 mM KCl, 2 mM dithiothreitol, 3% (vol/vol) glycerol, 7 mM $MgCl_2$, 5 μM ATP, 2.5 μCi of [γ-^{32}P]ATP, 50 ng of *Nde* I-digested pDN-AdML DNA (5), 20 ng of δ (fraction 35 of TSK SP-5-PW HPLC shown in Fig. 3*D*), and 0.001 unit of rat liver RNA polymerase II. Reactions were stopped by addition of an equal volume of SDS sample buffer [100 mM Tris/HCl (pH 6.8) with 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, and 20% glycerol]. Phosphorylated proteins were analyzed by electrophoresis through SDS/polyacrylamide gels (6% or 15% acrylamide) (37).

RESULTS

Transcription Factor δ Has an Associated Protein Kinase Activity That Phosphorylates the Largest Subunit of RNA Polymerase II. Incubation of purified transcription factor δ with RNA polymerase II, DNA, and [γ-^{32}P]ATP results in formation of a phosphorylated species that exhibits a decreased electrophoretic mobility relative to that of the largest subunit of RNA polymerase II (Fig. 1*A*). Formation of the phosphorylated species depends on the presence of δ, RNA polymerase II, and Mg^{2+} and is strongly stimulated by DNA (Fig. 1*B*). Phosphorylation is stimulated by a variety of DNAs, including pDN-AdML (5), single-stranded M13, poly(dT), and poly(dI)·poly(dC) (Fig. 1*B* and data not shown).

To provide evidence that the phosphorylated species is in fact the largest subunit of RNA polymerase II, immunoprecipitation experiments were performed. As shown in Fig. 2, this species can be immunoprecipitated with a monoclonal antibody, 8WG16 (18, 38), which was raised against wheat germ RNA polymerase II and is specific for the CTD of the largest polymerase subunit. The phosphorylated species is not precipitated with a control monoclonal antibody, HB22, of the same isotype. Further evidence that the phosphorylated species is the largest subunit of RNA polymerase II comes from the observation that it comigrates in SDS/polyacrylamide gels with a species recognized in Western blots by 8WG16 (data not shown).

As shown in Figs. 3 and 4, kinase activity copurifies with transcription activity when δ is analyzed by high-resolution ion-exchange HPLC on TSK SP-5-PW and TSK DEAE-5-PW and by sucrose gradient sedimentation, supporting the contention that transcription factor δ has a closely associated protein kinase activity. As described previously, the most highly purified preparations of δ contain a set of polypeptides

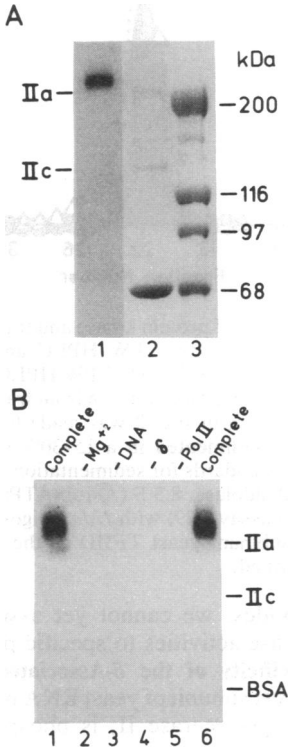

FIG. 1. Purified preparations of δ contain protein kinase activity. (*A*) Autoradiogram of an SDS/6% polyacrylamide gel shows products of the protein kinase reaction (lane 1). Coomassie blue-stained SDS/6% polyacrylamide gel shows rat liver RNA polymerase II containing bovine serum albumin (BSA) at 0.5 mg/ml (lane 2) and high molecular weight protein standards (Bio-Rad) (lane 3). (*B*) Requirements for protein kinase activity. Protein kinase assays were performed as described in *Materials and Methods* except that $MgCl_2$, DNA, δ, or RNA polymerase II (Pol II) was omitted from reaction mixtures as indicated. Forty nanograms of δ (fraction VI; ref. 29) was used in reactions, which were incubated for 3 min at 28°C.

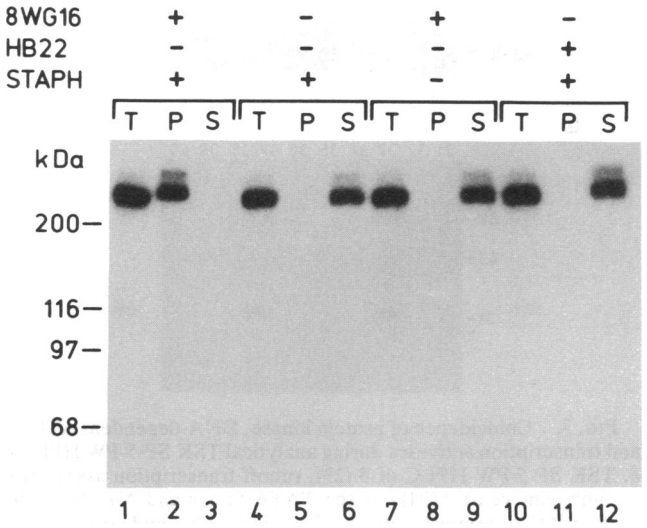

FIG. 2. The largest subunit of RNA polymerase II is phosphorylated. Protein kinase assays were performed as described. After a 60 min incubation, a 2-μl aliquot of each total reaction mixture was removed and diluted with SDS sample buffer. As indicated, ≈1 μg of either 8WG16 or HB22 antibody was added to the remaining portions of the reaction mixtures. After a further 30-min incubation at 4°C, 2 μl of formalin-fixed *Staphylococcus aureus* (Bethesda Research Laboratories), which had been washed in 40 mM Hepes/NaOH (pH 7.9) containing 80 mM KCl and 0.5 mg of bovine serum albumin per ml, was added to reaction mixtures as indicated (STAPH), and the mixtures were incubated at room temperature for 5 min. Reaction mixtures were then centrifuged at 3000 × *g* for 2 min, and the supernatant was removed and diluted with SDS sample buffer. Pellets were washed once with 60 μl of 20 mM Hepes/NaOH (pH 7.9) containing 20% glycerol, 0.1 M KCl, 1 mM EDTA, 1 mM dithiothreitol, and 0.5 mg of bovine serum albumin per ml and then were suspended in SDS sample buffer. Equivalent amounts of total reaction mixtures (T), immunoprecipitated pellets (P), and supernatants (S) were analyzed by SDS/6% polyacrylamide gel electrophoresis.

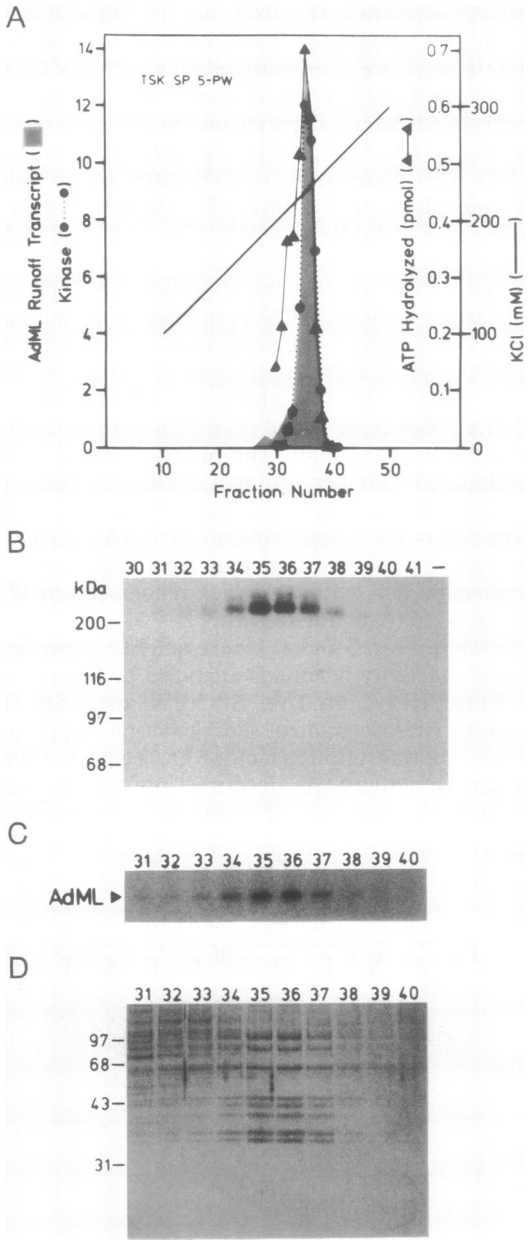

FIG. 3. Coincidence of protein kinase, DNA-dependent ATPase, and transcription activities during analytical TSK SP-5-PW HPLC of δ. TSK SP 5-PW HPLC of δ (29), runoff transcription assays with recombinant yeast TFIID as the TATA factor and *Nde* I-digested pDN-AdML as template (39), ATPase assays (29), and protein kinase assays (*Materials and Methods*) were performed as described. Elution profiles of protein kinase, DNA-dependent ATPase, and transcription activities during TSK SP-5-PW HPLC are shown in *A*. "Kinase" refers to the relative incorporation of ^{32}P into the largest subunit of RNA polymerase II (expressed in arbitrary units determined by densitometry of appropriate exposures of the autoradiogram shown in *B*). Here and in Fig. 3, "AdML Runoff Transcript" refers to the relative synthesis, per transcription reaction, of runoff transcripts synthesized from the adenovirus major late promoter in pDN-AdML (expressed in arbitrary units determined by densitometry of autoradiograms). The amount of AdML runoff transcript synthesized in this experiment was determined by densitometry of the autoradiogram shown in *C*. Aliquots of TSK SP-5-PW column fractions were analyzed by SDS/8% polyacrylamide gel electrophoresis, and protein was visualized by silver staining (*D*).

ranging in size from 94 to 35 kDa (refs. 29 and 33 and Fig. 3*D*, fractions 35 and 36), which copurify with transcription, kinase, and DNA-dependent ATPase activities. Because we have been unable to reconstitute any of these activities from

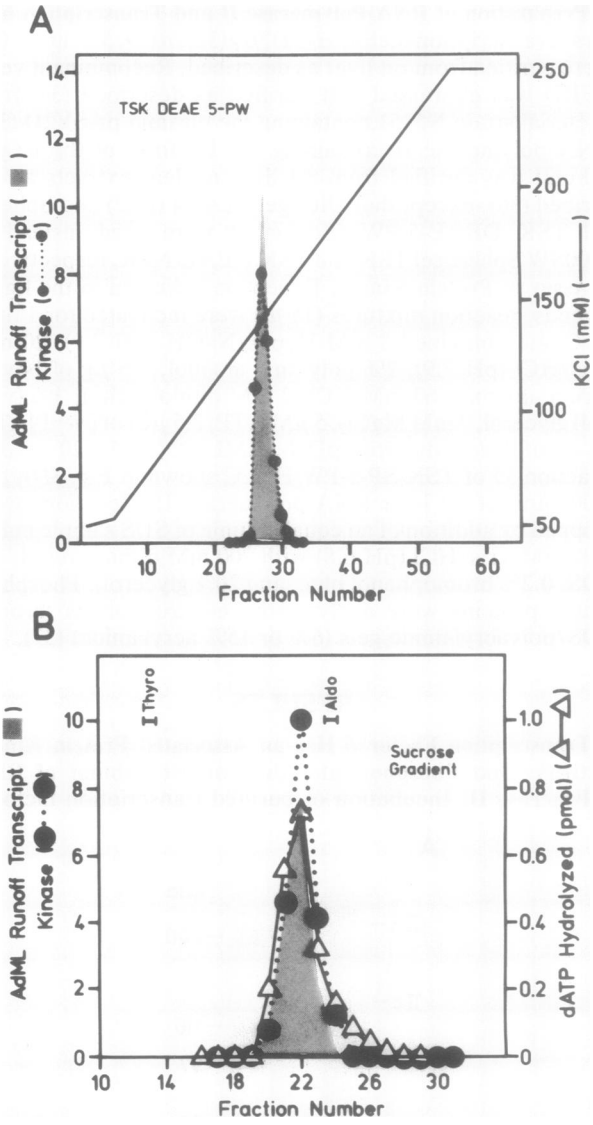

FIG. 4. Coincidence of protein kinase and transcription activities during analytical TSK DEAE-5-PW HPLC and sucrose gradient sedimentation of δ. (*A*) TSK DEAE 5-PW HPLC was performed as described (29). (*B*) An 80-μl aliquot of δ from fraction 34 of the TSK SP-5-PW column shown in Fig. 2 was made 0.5 mg/ml in bovine serum albumin and sedimented in a 15–30% sucrose gradient as described (34). The standards for sedimentation were thyroglobulin, 19.2 S (Thyro), and aldolase, 8.3 S (Aldo). ATPase assays (29), and runoff transcription assays (39) with *Nde* I-digested pDN-AdML as template and recombinant yeast TFIID as the TATA factor were performed as described.

isolated polypeptides, we cannot yet assign transcription, kinase, and ATPase activities to specific polypeptides.

Substrate Specificity of the δ-Associated Protein Kinase Activity. The largest subunit of yeast RNA polymerase II, like that of rat RNA polymerase II, is phosphorylated by the δ-associated kinase activity in a reaction stimulated by DNA. Although neither casein nor histones are measurably phosphorylated under these conditions, a synthetic peptide containing three copies of the carboxyl-terminal heptapeptide repeat of the largest subunit of RNA polymerase II is phosphorylated (Fig. 5*A*), consistent with the idea that the δ-associated kinase will phosphorylate the CTD of the largest subunit of RNA polymerase II. At the present time, we cannot rule out the possibility that other sites within the largest subunit are also phosphorylated. Surprisingly, phosphorylation of the CTD peptide is unaffected by DNA; when

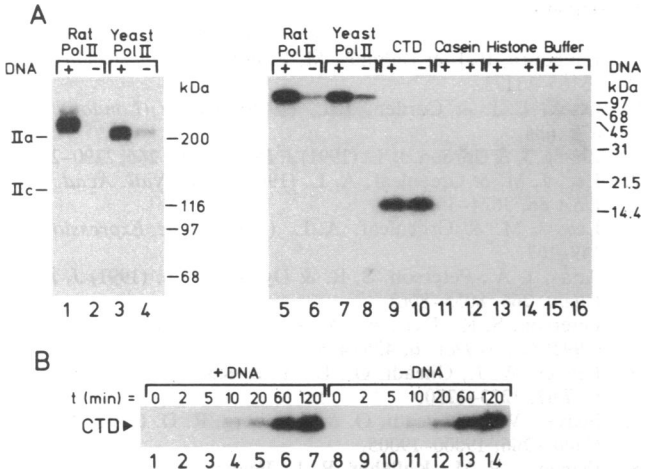

FIG. 5. Substrate specificity of protein kinase activity. (*A*) Substrates for protein kinase assays were ≈50 ng of immunoaffinity-purified yeast RNA polymerase II (40) (lanes 3, 4, 7, and 8), ≈2 μg of a synthetic peptide containing three copies of the carboxyl-terminal heptapeptide repeat (CTD) (lanes 9 and 10), ≈2 μg (lane 11) or ≈4 μg (lane 12) of casein (partially dephosphorylated, Sigma), and ≈2 μg (lane 13) or ≈4 μg (lane 14) of histones (lysine-rich fraction, Sigma type III-S). SDS/polyacrylamide gel electrophoresis was carried out in either 6% (lanes 1–4) or 15% (lanes 5–16) gels. IIa and IIc indicate the electrophoretic mobilities of the IIa and IIc subunits of rat RNA polymerase II in a stained marker lane. (*B*) Protein kinase assays were performed with 1 μg of the CTD peptide; reaction volume was 7.5 μl. Reaction mixtures were incubated for the times indicated.

the CTD peptide is used as substrate, the kinetics of phosphorylation in the presence and absence of DNA are indistinguishable (Fig. 5*B*).

Our evidence suggests that both ATP and GTP can function as phosphate donors in phosphorylation of the largest subunit of RNA polymerase II. Phosphorylation of the largest polymerase subunit is inhibited by addition of a 400-fold molar excess of ATP, dATP, or GTP but is not significantly affected by addition of the same levels of either CTP or UTP (Fig. 6*A*). ATP, however, appears to be utilized more efficiently than GTP; the rate of phosphorylation is ≈10-fold

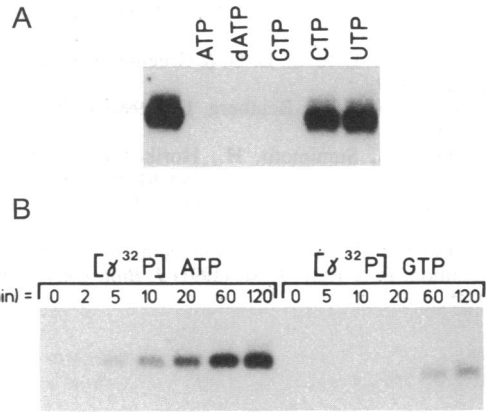

FIG. 6. Nucleotide specificity of protein kinase activity. (*A*) Protein kinase assays were performed as described in *Materials and Methods* except that, in addition to 5 μM ATP and 2.5 μCi of [γ-^{32}P]ATP, the nucleotide compositions of reaction mixtures were as follows: 2 mM ATP (lane 2), 2 mM dATP (lane 3), 2 mM GTP (lane 4), 2 mM CTP (lane 5), and 2 mM UTP (lane 6). (*B*) Protein kinase assays were performed as described in *Materials and Methods* except that reaction mixtures in lanes 8–14 contained 5 μM GTP and 2.5 μCi of [γ-^{32}P]GTP. Reaction mixtures were incubated for the times indicated.

greater in the presence of 5 μM ATP than in the presence of 5 μM GTP (Fig. 6*B*).

DISCUSSION

We have been engaged in studies to define the role(s) of ATP in promoter-specific initiation by RNA polymerase II in a reconstituted transcription system derived from rat liver. Our approach has been to resolve and purify the transcription factors required for initiation and to assess their abilities, individually and in combination, to hydrolyze or bind ATP. In addition to RNA polymerase II and the TATA factor, initiation in the rat liver system requires the action of four transcription factors designated α (31), βγ (32), δ (29, 33), and ε (34). Factors α, βγ, and ε, which are homologs of the human transcription factors TFIIB (41, 42), RAP30/74 (TFIIF) (43, 44), and TFIIE (45, 46), respectively, do not exhibit measurable ATP-hydrolyzing or -binding activities. We have observed, however, that δ possesses an associated DNA-dependent ATPase activity (29), suggesting that it could play a role in ATP-dependent activation of transcription.

As described in this report, further investigation of δ has revealed that it also possesses a closely associated protein kinase activity capable of catalyzing phosphorylation of the carboxyl-terminal heptapeptide repeat found in the largest subunit of RNA polymerase II. We do not know which residues in the largest polymerase subunit are targets for phosphorylation by the δ kinase. Further, although we have not determined the number of phosphoryl groups added by the δ kinase, phosphorylation of the largest polymerase subunit results in a measurable shift in its electrophoretic mobility (Fig. 1*A*, compare lanes 1 and 2), indicating phosphorylation at multiple sites (8). In any case, it is unlikely that the δ kinase alone is responsible for complete conversion of the largest polymerase subunit to the fully phosphorylated (IIo) form. Evidence indicates that, in mammalian cells, the CTD can be phosphorylated *in vivo* on both serine and threonine residues (21), whereas we detect phosphorylation of the largest polymerase subunit and the CTD peptide by the δ kinase only on serine residues (data not shown). Consequently, it is likely that multiple CTD kinases, such as those previously identified in yeast (22, 23), mouse (20, 21), and human (19, 24, 25) cells, play an important role in regulating the phosphorylation state of RNA polymerase II.

The mechanism by which DNA stimulates phosphorylation of the largest subunits of rat and yeast RNA polymerase II has not been defined. It is possible, for example, that the δ-associated kinase is stimulated through a direct interaction with DNA. Alternatively, it is possible that when bound to DNA, RNA polymerase II is a better substrate for kinase activity. The observation that phosphorylation of the CTD peptide is not stimulated by DNA is consistent with the latter possibility.

Because δ possesses both associated CTD kinase and DNA-dependent ATPase activities, it resembles yeast RNA polymerase II transcription factor b (27), which has also been shown to be associated with CTD kinase and DNA-dependent ATPase activities (26). Unlike the δ-associated kinase, however, the factor b kinase is not capable of utilizing GTP as a phosphate donor, nor is it reported to be stimulated by DNA (26). It is not clear how δ relates to other known mammalian transcription factors, although the polypeptide compositions of highly purified preparations of δ and the human transcription factor BTF2 (47) are similar. Furthermore, it is not clear how the δ-associated kinase relates to previously described protein kinases capable of phosphorylating the CTD (19–25). Because the δ kinase is stimulated by DNA, it is possible that it may be related to the partially purified "template-associated" or DNA-stimulated CTD kinases that have been characterized in several laboratories

(19, 24, 25). These kinases, however, have not been shown to be associated with transcription factor activity. In addition, the δ kinase appears to be distinct from mammalian CTD kinases containing $p34^{cdc2/CDC28}$ (20, 21), since antibodies specific for p34 do not crossreact with any of the polypeptides contained in the most highly purified preparations of δ (data not shown).

In conclusion, the observation that RNA polymerase II transcription factors from both yeast and mammalian cells are closely associated with kinase activity capable of phosphorylating the heptapeptide repeat of the CTD is consistent with the model that phosphorylation of the CTD plays an important role in transcription. Nonetheless, it has not been demonstrated that the specific phosphorylation events catalyzed by the factor b- or δ-associated kinases function in either basal or activated transcription, and it will be important to address these questions in future studies.

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