Partial Purification and Characterization of Two Distinct Protein Kinases That Differentially Phosphorylate the Carboxyl-terminal Domain of RNA Polymerase Subunit IIa*

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RNA polymerase II is a multisubunit enzyme composed of two large subunits of molecular weight in excess of 100,000 and a collection of 8–10 smaller subunits. The largest subunit, designated IIa, contains at its carboxyl terminus a highly repetitive domain consisting of tandem repeats of the consensus sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser. Extensive phosphorylation within this COOH-terminal domain (CTD) gives rise to subunit IIo which has a markedly reduced mobility in SDS-polyacrylamide gel electrophoresis (PAGE) relative to subunit IIa. Recent evidence suggests that RNA polymerase IIa, containing an unphosphorylated CTD, is involved in preinitiation complex assembly, whereas RNA polymerase IIo is involved in elongation. Consequently, CTD phosphorylation is thought to occur after RNA polymerase II has bound to the promoter by a protein kinase that stably associates with the preinitiation complex.

We present here the partial purification and characterization of two distinct CTD kinases from a HeLa cell transcription extract. These CTD kinases, designated CTDK1 and CTDK2, are fractionated by chromatography on Mono Q. CTDK1 catalyzes the incorporation of approximately 33 pmol of phosphate/pmol of calf thymus RNA polymerase subunit IIa, almost exclusively on serine. CTDK2 catalyzes the incorporation of approximately 50 pmol of phosphate/pmol of calf thymus subunit IIa, predominantly on serine; appreciable phosphate transfer onto threonine is also observed. Phosphorylation by CTDK2, but not CTDK1, results in a complete mobility shift in SDS-PAGE of subunit IIa to the position of IIo. CTDK1 can utilize ATP, ADP, or CTP as phosphate donor, whereas CTDK2 can utilize only ATP or dATP. The apparent $K_m$ for ATP is 30 pmol for CTDK1 and 60 pmol for CTDK2. CTDK1 and CTDK2 also differ in their protein substrate specificity. CTDK1 phosphorylates casein whereas CTDK2 does not. Neither kinase phosphorylates phosvitin or histone H1 to an appreciable extent. CTDK1 and CTDK2 do not appear to be related to cdc2 kinases as determined by their inability to phosphorylate H1 and their failure to react with antibodies directed against the cdc2 kinase. These results establish that a partially fractionated HeLa transcription extract contains two distinct CTD kinases that differ in their nucleotide requirements and in their patterns of CTD phosphorylation.

The largest subunit of RNA polymerase II (RNAP II) contains at its COOH terminus a highly repetitive domain consisting of tandem repeats of the 7-amino acid consensus sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser, designated the CTD (Allison et al., 1988; Corden et al., 1985; for review, see Corden, 1990). The heptapeptide repeat is present in 52 copies in mammals, 42 copies in Drosophila, and 26–27 copies in yeast. Each repeat contains multiple potential phosphorylation sites, and indeed RNAP II can be found in both the nonphosphorylated and highly phosphorylated forms in vivo and in vitro. The unphosphorylated form of the largest subunit is designated IIa, whereas the phosphorylated form of this subunit is designated IIo. The enzymes containing these subunits are designated RNAP IIa and RNAP IIO, respectively. The results of a variety of in vitro experiments indicate that the form of the enzyme that recognizes the promoter is RNAP IIa (Laybourn and Dahmus, 1989, 1990; Lu et al., 1991; Chesnut et al., 1992). RNAP IIO, containing the hyperphosphorylated CTD, is the predominant form in exponentially growing HeLa cells and appears to be involved in transcript elongation (Kim and Dahmus, 1986; Bartholomew et al., 1986; Cadena and Dahmus, 1987; Payne et al., 1989).

Genetic analysis has established that the CTD is essential for viability (Allison et al., 1988; Bartolomei et al., 1988; Zehring et al., 1988). Furthermore, a difference in sensitivity in the expression of specific genes to the length of the CTD has been established in yeast (Nonet et al., 1987; Scafe et al., 1990). These studies imply a gene-specific role for the CTD in the expression of at least some eukaryotic genes. In vitro studies of deletions within the CTD indicate that this domain is important for initiated complex formation (Liao et al., 1991).

Protein kinases that phosphorylate the CTD in vitro have been identified and purified to varying degrees from a number of eukaryotes, including yeast (Lee and Greenleaf, 1989; Feaver et al., 1991), Aspergillus (Stone and Reinberg, 1992), wheat (Guilfoyle, 1989), mouse (Cisek and Corden, 1991), and man (Payne et al., 1989; Legagneux et al., 1990; Arias et al., 1991; Lu et al., 1991). Genes encoding a CTD kinase from yeast and a cdc2-related kinase from mouse have been cloned and sequenced (Lee and Greenleaf, 1991; Cisek and Corden, 1989). Deletion of each of these kinase genes in yeast, how-

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1 The abbreviations used are: RNAP, RNA polymerase; CTD, COOH-terminal domain; DRB, 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole; DTT, dithiothreitol; MPP, M-phase promoting factor; PAGE, polyacrylamide gel electrophoresis.
however, results in only a partial reduction in subunit IIa phosphorylation (Kolodziej et al., 1990; Lee and Greenleaf, 1991). Thus, these authors have suggested that there may well be additional protein kinases involved in regulating the phosphorylation state of the CTD.

In vitro studies that examine the state of CTD phosphorylation during specific stages of transcription support the idea that the phosphorylation/dephosphorylation cycle is integral to the transcription cycle (Laybourn and Dahmus, 1990; Chesnut et al., 1992). The observation that RNA polymerase IIa is involved in preinitiation complex assembly, whereas RNAP IIo is involved in elongation, indicates that the CTD kinase must associate directly with the preinitiation complex and that each round of transcription is associated with the reversible phosphorylation of the CTD. Indeed, the presence of a CTD phosphatase that converts RNAP IIo to IIa, and completes the cycle, has been reported recently (Chesnut et al., 1992). Precisely when the phosphorylation occurs relative to the initiation of a transcript, and the immediate consequences of this phosphorylation, are not known.

A detailed understanding of the relationship between CTD phosphorylation and transcription will require the purification of specific CTD kinases and an analysis of their activity in reconstituted transcription systems. Several CTD kinases have been purified from transcription extracts, and some of these appear to co-purify with essential general transcription factors (Payne et al., 1989; Fevery et al., 1991; Serizawa et al., 1992). Previous studies have also established that CTD kinases can be recovered as integral components of the preinitiation complex (Laybourn and Dahmus, 1990; Arias et al., 1991). Results presented in this study show that the original activity identified in HeLa transcription extracts (Payne et al., 1989) can be chromatographically resolved into two distinct CTD kinase activities. These CTD kinases differ with respect to the stoichiometry of RNAP II CTD phosphorylation and in their nucleotide and protein substrate specificities.

**Experimental Procedures**

**Materials**—Ultrapure nucleotides were purchased from Pharmacia LKB Biotechnology Inc. Radiolabeled [γ-32P]ATP was obtained from Amersham Corp. Heparin-Sepharose CL-4B was prepared by the method of Teissere et al. (1977). The Mono Q and Mono S HR 5/5 columns were obtained from Pharmacia LKB Biotechnology Inc. Phosvitin was purchased from Sigma. Casein was obtained from Nutritional Biochemicals and treated as described by Reimann et al. (1971). Purified MF from Xenopus and antibodies directed against the cdc2 kinase were kindly provided by James Muller (University of Colorado, Denver, CO) and Jean Wang (University of California, San Diego, CA), respectively. Histone H1 was kindly provided by Thomas Langan (University of Colorado, Denver, CO).

**Buffers**—Buffer A contained 20 mM Tris-HCl, pH 7.9, 0.1 mM EDTA, 0.5 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride, and 20% glycerol. Buffer B contained 50 mM Tris-HCl, pH 7.9, 5 mM MgCl₂, 0.5 mM DTT, and 20% glycerol. Buffer C contained 50 mM Tris-HCl, pH 7.9, 0.1 mM EDTA, 0.5 mM DTT, and 10% glycerol. Buffer D was the same as buffer A except that it did not contain phenylmethylsulfonyl fluoride.

**Assays for CTD Kinase Activity**—Two distinct assays were used for the determination of CTD kinase activity. The first assay is based on the mobility shift in SDS-PAGE of subunits IIa to the position of subunit IIo that results from phosphorylation of the CTD (Payne et al., 1989). The second assay is based on the direct transfer of [γ-32P]ATP from [γ-32P]ATP to subunit IIa. The concentration of [γ-32P]ATP contained below 25 mM by dilution or dialysis for all CTD kinase assays.

**Mobility Shift Assay**—Purified calf thymus RNAP IIa was labeled by phosphorylation with casein kinase II in the presence of [γ-32P]ATP (Payne et al., 1989). [γ-32P]-Labeled RNAP IIa was incubated with fractions containing CTD kinase in the presence of 17 mM Tris-HCl, pH 7.9, 8 mM MgCl₂, 25 μM EDTA, 0.01% Triton X-100, 0.5 mM DTT, and 10% glycerol for 10 min at 30 °C in a final reaction volume of 25 μl. Reactions were stopped by the addition of 5 μl of 6×loading buffer (0.375 M Tris-HCl, pH 6.8, 12% SDS, 30% β-mercaptoethanol, 0.065% bromphenol blue), and labeled proteins were resolved by electrophoresis on a 5% polyacrylamide SDS gel according to the procedure described by Laemmli (1970). CTD kinase activity during early stages of purification, including the phosphocellulose P11 and heparin-Sepharose columns, was routinely determined by the mobility shift assay.

**32P Incorporation Assay**—Except as noted in the text, reaction mixtures contained CTD kinase, 0.032 μg of purified RNAP IIa, 10 μM [γ-32P]ATP (4 Ci/mmol), 17 mM Tris-HCl, pH 7.9, 8 mM MgCl₂, 25 μM EDTA, 0.01% Triton X-100, 0.5 mM DTT, and 10% glycerol in a final reaction volume of 25 μl. Reactions were incubated at 30 °C for 10 min, stopped by the addition of sample loading buffer, and resolved by SDS-PAGE as described above. The gel was dried, and [32P] incorporation into the largest subunit of RNAP II was quantitated on a Betascope 603 Bio analyzer (Betagen). RNAP IIa used in experiments to determine stoichiometry was purified by a modification of the procedure of Kim and Dahmus (1988). RNAP IIa purified by this method shows a single IIa band in SDS-PAGE as opposed to the doublet generally observed. Purified RNAP IIa for other experiments was obtained by the method of Hodo and Blatti (1977) as modified by Laybourn and Dahmus (1990). One unit of CTD kinase activity is defined as the amount of enzyme that catalyzes the transfer of 1 pmol of phosphate from ATP to RNAP subunit IIa/min under the conditions described.

**Purification of CTD Kinases from HeLa Cells**—HeLa whole cell extract was prepared from HeLa cells grown according to the method of Weil et al. (1979) as modified by Dahmus and Kelinger (1983). The S-100 extract, approximately 750 μg of protein from 4 × 10⁶ HeLa cells, was dialyzed against buffer A containing 0.1 M KCl, centrifuged at 27,000 × g for 30 min, and filtered through Miracloth. The extract was then loaded onto a phosphocellulose P11 column (25 μg of protein/ml of resin) equilibrated previously in buffer A containing 0.1 M KCl. After washing with 3 column volumes of buffer A containing 0.1 M KCl, the column was sequentially developed with 3 column volumes of buffer A containing 0.5 and 1.0 M KCl. In the initial experiments, an additional wash with 2 column volumes of buffer A containing 0.1 M KCl was included. CTD kinase activity was recovered in both the 0.3 and 0.5 M KCl eluant (Fig. 1). Fraction 0.5 M KCl peak were pooled (about 300 mg protein), diluted to 0.24 M KCl with buffer B, and loaded onto a heparin-Sepharose column (3 μg of protein/ml of resin) equilibrated previously in buffer B containing 0.15 M KCl. The column was washed with 3 column volumes of buffer B containing 0.24 M KCl and developed with 3 column volumes of buffer B containing 0.6 M KCl. Fractions of the 0.6 M KCl peak were pooled (about 150 mg protein), diluted to 0.15 M KCl with buffer B, and loaded onto a Mono Q column (3 μg of protein/ml of resin) equilibrated previously in buffer B containing 0.24 M KCl. The column was washed with 3 column volumes of buffer B containing 0.15 M KCl and loaded with a Mono S column eluted containing 0.15 M KCl. CTDKl was eluted at 0.22 M KCl, and CTDK2 at 0.27 M KCl, respectively. Approximately 2 × 10⁶ units for CTDKl and 1 × 10⁶ units for CTDK2 were recovered following fractionation on Mono Q. Further purification of both activities was achieved by chromatography on Mono S. CTDKl and CTDK2 were eluted at 0.1 M KCl by the addition of buffer C and loaded onto a Mono Q column eluted previously in buffer C containing 0.15 M KCl. Alternatively, the 0.6 M KCl peak from the heparin-Sepharose column was diluted to 0.15 M KCl and loaded directly onto Mono Q. The column was washed with 3 ml of buffer C containing 0.15 M KCl and developed with a 15-mL linear gradient of 0.15-0.4 M KCl in buffer C. CTDKl and CTDK2 eluted at 0.25 M KCl and 0.28 M KCl, respectively. Approximately 2 × 10⁶ units for CTDKl and 1 × 10⁶ units for CTDK2 were recovered following fractionation on Mono Q. Further purification of both activities was achieved by chromatography on Cibacron blue-agarose. Mono Q-purified CTDKl (1.8 mg) was loaded on a 4-ml column of Cibacron blue 3GA-agarose (Sigma) equilibrated previously with buffer D containing 0.3 M KCl. The column was washed with 3 column volumes of buffer D containing 0.3 M KCl. CTDKl was eluted with 3 column volumes of buffer D containing 0.6 M KCl.

Protein concentration was determined by the method of Bradford.
Electroeluted as described by Corden (1976) using bovine serum albumin as a standard. 

Analysis of Phosphoamino Acids—Phosphoamino acid analysis was performed as described by Cooper et al. (1983). RNA polymerase II (0.072 pmol), purified by a modification of the procedure of Kim and Dahmus (1988), was incubated in the presence of CTDK1 (0.3 unit) or CTDK2 (0.3 unit) and 50 μM [γ-32P]ATP (400 Ci/mmol) at 30 °C for 60 min as described above in a final reaction volume of 25 μl. Labeled subunit I 6o was purified by electrophoresis on a 5% polyacrylamide SDS gel according to the method of Laemmli (1970). The position of the 32P-labeled I 6o band was determined by autoradiography and excised from the gel using the autoradiogram as a template. Labeled subunit I 6o was electroeluted as described by Corden et al. (1985), except that the electroelution buffer also contained 10 mM EDTA and 10 mM EGTA to increase the stability of subunit I 6o. Electroeluted subunits were lyophilized and subjected to acid hydrolysis in 6 M HCl at 110 °C for 4 h. The liberated 32P-labeled phosphoamino acids were analyzed by two-dimensional thin layer electrophoresis. Samples (1 to 2 μl) were electrophoresed on polyethyleneimine cellulose thin layer plates in the first dimension in pH 1.9 buffer (50:156:1794, pyridine:glacial acetic acid:H2O) at 750 V for 30 min. This was followed by electrophoresis in the second dimension in pH 3.5 buffer (10:100:1890, pyridine:glacial acetic acid:H2O) at 550 V for 15 min. One μg each of phosphoserine, phosphothreonine, and phosphotyrosine was also included as internal standards. After electrophoresis, the plates were dried, and phosphoamino acids were visualized with ninhydrin. 32P-Labeled phosphoamino acids were visualized by autoradiography and quantitated on a Betagen analyzer.

RESULTS

Resolution of CTD Kinase 1 and CTD Kinase 2 by Chromatography on Mono Q—CTD kinase was purified from HeLa cell transcription extracts by chromatography on phosphocellulose P11, heparin-Sepharose, and Mono Q as described under “Experimental Procedures.” CTD kinase activity in Mono Q column fractions can be assayed by either the mobility shift assay or by the direct incorporation of 32P from [γ-32P]ATP. Columns prior to Mono Q were assayed by mobility shift. The extent of conversion of subunit IIa to I 6o, as determined by mobility shift in a 5% polyacrylamide SDS gel, is proportional to the CTD kinase activity. Both the degree of mobility shift from the position of IIa to that of I 6o and the amount of phosphate transferred to the largest subunit of RNA polymerase II can be assessed by the direct incorporation of 32P. The observations that no phosphate is incorporated into the largest subunit of RNA polymerase IIIB (data not shown), which lacks the CTD, and that the extent of phosphorylation of the largest subunit is proportional to the mobility shift in SDSPAGE (Cadena and Dahmus, 1987) indicate that these activities are true CTD kinases. In an effort to directly establish this point, RNA polymerase IIIA was phosphorylated in the presence of [γ-32P]ATP with CTDK1 and CTDK2, digested with limiting amounts of trypsin, and the resultant peptides analyzed by SDS-PAGE as described previously (Laybourn and Dahmus, 1989). Under these conditions, trypsin cleaves the CTD from subunit I 6o leaving the remainder of that subunit (IIIb) and the CTD fragment otherwise intact. The observations that label incorporated into subunit I 6o is recovered in a diffuse series of bands corresponding in position to the free phosphorylated CTD and that no label is observed in the position of subunit IIIb provide additional evidence that the sites of phosphorylation are confined to the CTD (data not shown).

As shown in Fig. 1, and as reported previously (Payne et al., 1989), CTD kinase activity can be readily detected in the crude S-100 transcription extract. Chromatography on phosphocellulose results in a broad elution pattern of detectable CTD kinase activity. Although the major fraction of CTD kinase activity elutes at 0.5 M KCl, significant amounts of activity are also found in the 0.3 M KCl and 1.0 M KCl protein peaks (Fig. 1). The activity recovered in the 0.3 M KCl eluate chromatographs on heparin-Sepharose, Mono Q, and Mono S columns in a manner indistinguishable from the activity recovered in the 0.5 M KCl peak. The broad distribution of CTD kinases may result from an overloading of the phosphocellulose column, the presence of distinct but related CTD kinases, or the association of CTD kinase with other factors that influence chromatography on phosphocellulose. The observation that the CTD kinase activities derived from the 0.3 and 0.5 M KCl peaks chromatograph similarly on Mono Q and Mono S suggested that the consolidation of these elutions from the phosphocellulose column into a single 0.5 M KCl elution. Purification of the 0.5 M KCl P11 peak by chromatography on heparin-Sepharose and Mono Q is shown in Fig.
1. Two peaks of CTD kinase activity are resolved on Mono Q. The first peak, designated CTD kinase 1 (CTDK1), elutes at approximately 0.23 M KCl, whereas the second peak, designated CTD kinase 2 (CTDK2), elutes at approximately 0.28 M KCl. No effect on activity was observed when aliquots of intervening fractions were incubated with either kinase (data not shown). The lack of an apparent shift in reactions containing the input fraction ("I") is due to dilution of the heparin-Sepharose peak fraction prior to chromatography on Mono Q. It is of interest to note that the maximum mobility shift catalyzed by CTDK1 appears to be less than the mobility shift catalyzed by CTDK2. Additional purification can be achieved by chromatography on Mono S as described under "Experimental Procedures" and shown in Fig. 1. Although CTDK1 and CTDK2 have been purified extensively from the S100 extract, a multiplicity of bands are apparent on a stained SDS gel. The activities of CTDK1 and CTDK2 following chromatography on Mono S are highly unstable. Whether this is due to an inherent property of the kinases, the loss of a stabilizing factor, or an instability to freeze-thaw is not known.

**Determination of the Stoichiometry of CTD Phosphorylation by CTD Kinase 1 and CTD Kinase 2**—In an effort to define the extent of phosphorylation necessary for the conversion of subunit IIa to IIo, the stoichiometry of phosphorylation of highly purified RNAP IIA with each CTD kinase was determined. Partially purified CTDK1 and CTDK2 contain proteins with mobilities in SDS-PAGE comparable with that of subunits IIo and IIa, respectively (left panel of Fig. 2A; for CTDK1, compare lanes 1 and 2; for CTDK2, compare lanes 3 and 4). Consequently, limiting amounts of CTD kinases were used in these experiments so that subunits IIa and IIo could be visualized directly by silver staining. Incubation of purified RNAP IIA in the presence of ATP and CTDK1 or CTDK2 results in a time-dependent shift in the mobility of subunit IIa to a position approximating that of IIo (Fig. 2A, right panel). It is important to note that the IIa subunit of RNAP IIA is quantitatively converted to the phosphorylated form by incubation with either CTD kinase. The maximum extent of shift is below that of IIo, especially in reactions catalyzed by CTDK1. The absence of unphosphorylated subunit at the end of the phosphorylation reaction indicates that this approach provides an accurate method for quantitation of the number of phosphates incorporated into the largest subunit under these conditions. The incorporation of phosphate into subunit IIa as a function of time is shown in the autoradiogram in Fig. 2B. A quantitation of the phosphate incorporated is shown in Fig. 2C. The incorporation of only 30% of the maximum level of phosphate results in a shift in subunit IIa mobility 80% of the distance to the position of IIo (compare lane 4 with lane 7 and lane 10 with lane 14). This result indicates that the mobility shift is not linear with respect to the incorporation of phosphate. Consequently, the apparent $K_m$ based on the mobility shift (Laybourn and Dahmus, 1990) is not an accurate measure of the actual $K_m$ as determined by phosphate incorporation.

The level of phosphorylation in the presence of increasing concentrations of CTDK1 and CTDK2 is shown in Fig. 3. It is apparent from the autoradiogram presented in A (as well as Figs. 1 and 2) that CTDK1 and CTDK2 differ in the extent of the mobility shift that they catalyze. The phosphorylation of RNAP IIA by CTDK1 results in a form of IIo that has a mobility shift distinctly less than that of subunit IIo formed by incubation with a less pure fraction of CTD kinase (Fig. 3A, compare lanes 2 and 8). The phosphorylation of RNAP IIA by CTDK2, on the other hand, results in a shift that approximates the position of IIo (compare lanes 14 and 15).

Quantitation of these results shows that the maximum number of phosphates incorporated by CTDK1 per molecule of subunit IIa is approximately 33. A range of 25–40 pmol of phosphate/pmol of RNAP IIA has been observed in different experiments. The number of phosphates incorporated by CTDK2 per molecule of subunit IIa is approximately 50, although these conditions do not appear to be saturating for CTDK2. A range of 40–60 pmol of phosphate/pmol of RNAP IIA has been observed in different experiments. The maximum extent of phosphorylation by CTDK2, therefore, amounts to about one phosphate/heptapeptide repeat. In the presence of 0.45 unit of both CTDK1 and CTDK2, there does not appear to be an increase in the stoichiometry of CTD phosphorylation beyond that observed in the presence of CTDK2 alone (data not shown).

**Identification of the Amino Acids Phosphorylated by CTD Kinase 1 and CTD Kinase 2**—The analysis of in vivo $^{32}$P-labeled HeLa cell RNAP II has established that the CTD is phosphorylated primarily on serine, with some phosphoryla-
tion of threonine (Cadena and Dahmus, 1987). In an effort to establish whether or not a correlation exists between the in vitro and in vivo reactions, the amino acids phosphorylated by CTDK1 and CTDK2 were determined. As can be seen in Fig. 4, the analysis of phosphoamino acids recovered following the partial hydrolysis of the largest subunit labeled by CTDK1 suggests that serine is the primary site of phosphorylation. The primary site of phosphorylation by CTDK2 also appears to be serine, although a significantly greater amount of phosphothreonine is observed. Under these conditions, the ratio of phosphoserine to phosphothreonine is approximately 30:1 for CTDK1 and approximately 9:1 for CTDK2. Phosphothreonine was not detected in these experiments nor was it observed when the hydrolysis time was reduced to 2 h (data not shown). These results are in agreement with the pattern of phosphorylation observed in in vivo labeled RNA II and are consistent with the idea that CTDK1 and CTDK2 play a role in the in vivo phosphorylation of RNA II. These results also suggest that CTDK1 and CTDK2 may recognize different target sequences within the CTD. In this regard it is interesting to note that, in the mouse CTD, position 2 of the hepta-peptide repeat is occupied by serine in 42 repeats and by threonine in 8 repeats, whereas position 5 is occupied by serine in 51 of the 52 repeats.

Substrate Specificity of CTD Kinase 1 and CTD Kinase 2—The nucleotide substrate specificity of CTDK1 and CTDK2 was examined by incubating 32P-labeled RNA II in the presence of increasing concentrations of ATP, dATP, or GTP with subsequent analysis by the mobility shift assay. The results presented in Fig. 5 establish that CTDK1 can utilize as nucleotide substrate ATP, dATP, or GTP, whereas CTDK2 can utilize only ATP or dATP. This result supports the idea that CTDK1 and CTDK2 are distinct kinases. Although CTDK1 is capable of utilizing any of these nucleotides as substrate, the apparent Km for ATP seems to be lower than that for dATP or GTP. The apparent Km values of CTDK2 for ATP and dATP appear comparable and higher than that of CTDK1 for ATP. As noted earlier, however, the apparent Km determined by the mobility shift assay is likely to be an underestimate of the actual Km. The apparent Km of both CTDK1 and CTDK2 for ATP has also been determined by the standard 32P incorporation assay and is approximately 30 μM for CTDK1 and 60 μM for CTDK2.

The ability of CTDK1 and CTDK2 to phosphorylate casein and phosphovitin was also examined. Incubation of 0.1 unit of CTDK1 or CTDK2 with 20 pmoles of casein resulted in the incorporation of 0.062 or 0.002 pmoles of phosphate, respectively, whereas incubation with 15 pmoles of phosphovitin resulted in the incorporation of 0.010 and 0.006 pmoles of phosphate, respectively. Therefore CTDK1, but not CTDK2, can utilize casein as substrate. Neither CTDK1 nor CTDK2 phosphorylates phosphovitin to an appreciable extent.

CTDK Kinase 1 and CTD Kinase 2 Are Not Related to MPF—Two distinct CTD kinases have also been fractionated from mouse ascites cells by anion exchange chromatography (Cisek and Corden, 1991). These kinases actively phosphorylate histone H1, and each contains a small subunit of 34 kDa that is the homologue to the Schizosaccharomyces pombe cdc2 gene product. One kinase corresponds to MPF, whereas the other...
is the cdc2 kinase associated with a 58-kDa subunit. It was, therefore, of interest to examine the relationship of CTDK1 and CTDK2 to the cdc2 kinase. A comparison of the ability of CTDK1, CTDK2, and MPF to phosphorylate histone H1 and RNAP subunit IIa is shown in Fig. 6. At the concentration of enzymes employed here, the CTD kinase activity of MPF is approximately 10-fold less than the activity of CTDK1 or CTDK2 (A, compare lane 3 with lanes 1 and 2; quantitation shown in B). At these same enzyme concentrations, a marked reversal of activity is observed when H1 serves as substrate. In the presence of H1, the relative activity of MPF is about 100-fold greater than the activity of either CTDK1 or CTDK2 (A, compare lane 6 with lanes 4 and 5; quantitation shown in B). Therefore, the preferred substrate for CTDK1 and CTDK2 is RNAP II, whereas the preferred substrate for MPF is H1. These results establish that the protein substrate specificity of CTDK1 and CTDK2 is distinctly different from that of MPF.

Further evidence in support of the idea that CTDK1 and CTDK2 are not related to MPF comes from the observation that antibodies directed against the cdc2 kinase do not react with either CTDK1 or CTDK2. Twenty units of CTDK1 and CTDK2, which is 100 times the amount used in Fig. 6, and 9 units of MPF were resolved by SDS-PAGE, transferred to Immobilon-P (Millipore), incubated with antibody, and reacted with 125I-protein A as described previously (Kim and Dahmus, 1986). Although a positive reaction was observed with MPF, no reaction was observed in either the CTDK1 or CTDK2 lanes (data not shown).

**DISCUSSION**

Increasing evidence supports the idea that phosphorylation of the CTD of the largest RNAP II subunit plays an important role in the early phases of transcription. Since CTD phosphorylation occurs after RNAP II has associated with the promoter, this reaction is of potential regulatory significance. An understanding of the involvement of the CTD in transcription, and of the consequences of its phosphorylation, is dependent on the purification and characterization of the protein kinases involved. The results presented here indicate that at least two distinct CTD kinases, designated CTDK1 and CTDK2, are present in HeLa cell transcription extracts. The observations that neither kinase will phosphorylate RNAP IIB, lacking a CTD, that both catalyze phosphorylation of subunit IIa at multiple sites leading to a mobility shift, and that the phosphate incorporated into subunit IIo is recovered in the free CTD following limited proteolysis, establish that CTDK1 and CTDK2 phosphorylate the CTD. CTDK1 and CTDK2 are resolved by chromatography on Mono Q and differ both qualitatively and quantitatively in their phosphorylation of RNAP II. Phosphorylation by CTDK2 results in the incorporation of about 50 phosphates/CTD and a mobility shift to the position of subunit IIo. Phosphorylation by CTDK1 results in the incorporation of about 33 phosphates/CTD and an incomplete mobility shift. CTDK1 and CTDK2 also differ in activity relative to the phosphorylation of serine and threonine, in their nucleotide and protein substrate specificities and in their apparent $K_m$ for ATP. No additive or synergistic stimulation of phosphate incorporation is observed upon incubation of RNAP IIa with both CTD kinases. Additional experiments are necessary to establish whether or not RNAP IIO prepared in vitro by phosphorylation with CTDK1 and CTDK2 is functionally equivalent to the in vivo form of RNAP IIO.

The lack of appreciable phosphorylation of histone H1 by...
either CTDK1 or CTDK2 distinguishes these kinases from many other CTD kinases that have been described previously. Histone H1 kinase activity has been demonstrated in CTD kinase preparations from mouse (Cisek and Corden, 1989), heat-shocked HeLa cells (Legagneux et al., 1990), and in HeLa cell transcription extracts (Lu et al., 1991). A number of these protein kinases have been shown to be related to the cdc2 kinase (Cisek and Corden, 1991). MPF has also been identified as Hk2 in HeLa transcription extracts prepared by Lu et al. (1991). This activity originates in the 1.0 M KCl eluate from the phosphocellulose column, a fraction in which we also observe CTD kinase activity. The observations that the protein substrate specificities of CTDK1 and CTDK2 are markedly different from that of MPF and that antibodies directed against the cdc2 kinase do not react with CTDK1 or CTDK2 provide convincing evidence that these kinases are not related to the cdc2 kinase. Previously reported CTD kinase activities also do not phosphorylate H1 significantly: yeast CTK1 (Lee and Greenleaf, 1989, 1991), yeast transcription factor b (Feaver et al., 1991), and a CTD kinase from wheat germ (Guilfoyle, 1989). The observation that CTDK1 and CTDK2 are sensitive to 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) (data not shown), whereas yeast CTK1 is not, suggests that these kinases are not related. Furthermore, CTDK1 and CTDK2 do not appear to be related to either the DNA-activated kinase described by Peterson et al. (1992) or the factor 5 CTD kinase described by Serizawa et al. (1992) in that no stimulation of CTDK1 or CTDK2 activity was observed by the addition of DNA (data not shown). However, caution must be exercised in the interpretation of these experiments, since agents that increase the solubility of RNAP IIA or non-specifically bind both CTD kinase and RNAP II may anomalously affect kinase activity.

If phosphorylation of the CTD plays an essential role in the initiation phase of transcription, CTD kinase activity should be intrinsic to an essential transcription factor (Payne et al., 1989). This idea is supported by the observation that CTD kinase appears to associate stably with the preinitiation complex (Laybourn and Dahmus, 1990; Arias et al., 1989). The association of CTD kinase activity with essential transcription factors in yeast and rat liver has recently been reported (Feaver et al., 1991; Serizawa et al., 1992). Although these factors are highly purified, definitive experiments remain to be done to establish that the kinase activity associated with these fractions is essential for transcriptional activity. In general, these kinases do not appear to phosphorylate casein, phosvitin, or histones to an appreciable extent and are unable to utilize GTP efficiently as phosphate donor. An understanding of the relationship between these CTD kinases is dependent on a more complete characterization, including the molecular cloning of the gene(s) encoding specific kinases.

The existence of more than one CTD kinase that functions in vivo in the maintenance of CTD phosphorylation has been suggested by gene disruption experiments in yeast (Lee and Greenleaf, 1991). The relatively large number of protein kinases that phosphorylate the CTD in vitro is consistent with the idea that a multiplicity of protein kinases function in vivo in CTD phosphorylation. This multiplicity of CTD kinases may reflect the complexity of interactions in which the CTD participates. Proposed functions for the CTD include (a) facilitating the association of RNAP II with the promoter (Sigler, 1988; Lu et al., 1991; Chesnut et al., 1992), (b) facilitating the transition from preinitiation complex assembly to elongation (Laybourn and Dahmus, 1989, 1990), (c) facilitating the progression of RNAP II through nucleosomes (Corden et al., 1985), and (d) influencing pausing and termination (Spencer and Groudine, 1990; Shermoen and O’Farrel, 1991; Marshall and Price, 1992). The involvement of the CTD at these various steps may be dependent on the interaction of the CTD with discrete sets of transcription factors. These interactions may in turn be influenced by unique CTD kinases. Because little is known concerning the turnover of phosphate during a single round of transcription, the possibility that unique CTD kinases function at independent steps in the transcription reaction cannot be excluded. Consistent with this idea is the observation of Marshall and Price (1992) that a DRB-sensitive step exits early in the elongation phase of transcription. Alternatively, multiple CTD kinases may participate in controlling CTD function at a given step in the transcription cycle. The complex array of transcription factors that are involved in preinitiation complex assembly at a given promoter may, for example, influence the type of CTD kinase that associates with that promoter. This in turn could influence both the extent and nature of CTD phosphorylation in a way that could affect the efficiency of initiation as well as subsequent events including termination.

According to this view, distinct CTD kinase activities may control interactions between the CTD and transcription factors at multiple stages in the transcription cycle or differentially influence CTD interactions at a specific stage in transcription. One group of kinases (CTDK1, CTDK2, factor 5, Hkl) could act during the initiation phase of transcription, whereas other groups, such as that represented by MPF (Hk2), could participate in the control of other phases of transcription, such as elongation and termination. These considerations do not rule out the possibility that phosphorylation of the CTD by discrete protein kinases and interactions between the phosphorylated or unphosphorylated CTD and transcription factors may be combinatorial in nature. As reported here, CTD kinases independently induce different levels of phosphorylation within the CTD and on different combinations of target amino acid residues. These effects could conceivably create multiple charge densities and conformations within the CTD, thereby modulating interactions between the CTD and various components of the transcription apparatus.

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